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The Customizable E-cigarette Resistance Influences Toxicological Outcomes: Lung degeneration, inflammation, and oxidative stress-induced in a rat model

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The customizable e-cigarette resistance influences toxicological outcomes

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E-cigarette customization and toxicity

Keywords

Electronic cigarette, resistance, oxidative stress, inflammation, animal model.

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Abstract

Despite the knowledge gap regarding the risk-benefit ratio of the electronic cigarette (e-cig), its use has grown exponentially, even in teenagers. E-cig vapour contains carcinogenic compounds (e.g., formaldehyde, acetaldehyde and acrolein) and free radicals, especially reactive oxygen species (ROS) that cause toxicological effects, including DNA damage. The role of e-cig voltage customization on molecule generation has been reported, but the effects of the resistance on e-cig emissions and toxicity are unknown. Here we show that the manipulation of e-cig resistance influences the carbonyls production from non-nicotine vapour and the oxidative and inflammatory status in a rat model. Fixing the voltage at the conventional 3.5 V, we observed that the amount of the selected aldehydes increased as the resistance decreased from 1.5 to 0.25 Ω . Under these conditions, we exposed Sprague Dawley rats to e-cig aerosol for 28-days, and we studied the pulmonary inflammation, oxidative stress, tissue damage and blood homeostasis. We found a perturbation of the antioxidant and phase-II enzymes, probably related to the increased ROS levels due to the enhanced xanthine oxidase and P450-linked monooxygenases. Furthermore, frames from scanning electron microscope showed a disorganization of alveolar and bronchial epithelium in 0.25 Ω group. Overall, various toxicological outcomes, widely recognized as smoke-related injuries, can potentially occur in e-cig consumers who use low-voltage and resistance device. Our study suggests that certain “tips for vaping safety” cannot be established, and encourage further independent investigations to help public health agencies in regulating the e-cig use.

Introduction

Electronic cigarettes (e-cigarettes, e-cigs) have been distributed on the global market for almost a decade as both potential approach to aid smoking cessation (Franks et al., 2018) and safe alternative to combustion cigarettes. However, evidence on quit smoking remains inconclusive and, actually, the Centres for Disease Control and Prevention (CDC), Food and Drug Administration (FDA), and Georgia State University stated that the number of teens who had never smoked but use electronic cigarette increased three times during the period 2011-2013 (Lestari et al., 2018). Furthermore, a great deal of studies testifies its dangerousness. While in the early years it was extensively spread the message about e-cig safety, current literature reports a growing number of evidence on harmful outcomes deriving from the device use (Lerner et al., 2015; Sussan et al., 2015; McConnel et al., 2017; Cardenia et al., 2018; Vivarelli et al., 2019). E-cig vapour, same as tobacco smoke, induces carcinogen metabolizing enzymes and oxidative stress, which play a key role in the pathogenesis of chronic, inflammatory and degenerative diseases, including chronic obstructive pulmonary disease (COPD) and cancer (Lerner et al., 2015; Muthumalage et al., 2018; Scott et al., 2018). In fact, several

toxicological aspects closely related to cancer have been investigated in *in vitro* systems (Zhang et al., 2012; Scheffer et al., 2015; Scott et al., 2018; Vasanthi Bathrinarayanan et al., 2018) and confirmed in *in vivo* ones (Canistro et al., 2017; Lee et al., 2018).

E-cig consists of a mouthpiece, a refillable cartridge, a lithium battery and a heating atomizer. In the majority of the devices, a power button allows the user to activate the heating element during inhalation, thus producing the flavoured vapour. The wide variety of e-liquids on the market, together with the continuous evolution in the e-cig technologies, makes these devices extremely customizable. Although the e-liquid is generally composed by vegetable glycerol (VG), propylene glycol (PG), water and an impressive variety of flavours and nicotine at different concentrations, the personalization of the vaporization process is responsible for different emission levels of toxic and/or carcinogenic carbonyl compounds, such as formaldehyde, acetaldehyde, acrolein (Goniewicz et al., 2014; Bitzer et al., 2018) and reactive free radicals (Goel et al., 2015; Lerner et al., 2015, Sussan et al., 2015). The exposure to aldehydes derived from e-cig vapours is considered a risk factor for human health. In particular, formaldehyde and acetaldehyde are classified as Group 1 and Group 2B carcinogens, respectively, by the International Agency for Research on Cancer (IARC, 1999 and 2012). Acrolein is listed as hazardous air pollutant by the United States Environmental Protection Agency (U.S. EPA, 2003). Moreover, the generation of carbonyls is related to the formation of radicals (*e.g.*, hydroxyl radicals), which are responsible for the oxidation and fragmentation of glycols (Geiss et al., 2016) and the possibility to arbitrarily adjust the total power of the device by combining different voltage and resistance levels may have a considerable impact on human health (Chausse et al., 2015).

Contrary to the customary statements that carbonyl compounds are generated only when high voltage is applied (Jensen et al., 2015), it has been observed that carcinogenic aldehydes, such as formaldehyde, are produced even in lower power breath activated e-cig (Bitzer et al., 2019). Since the combination of applied voltage and resistance value of the filament coil is responsible for the device heating power through the Joule effect (Chausse et al., 2015), we believe that the extent of the toxicological effects can be strictly influenced by consumers' habits.

In this investigation, we have therefore set e-cig devices at a fixed voltage value (the most commonly used of 3.5 V) in order to determine whether application of low (0.25 Ω) and medium (1.5 Ω) coil resistances affects the carbonyls generation and the biological effects of the resulting non-nicotine vapour on the pulmonary oxidative and inflammatory status in a rat model. Lung damage and blood homeostasis were also studied.

Material and methods

E-cigarette, liquid refill and device settings

A commercially available e-cig (Eleaf™ Pico) powered by a rechargeable lithium battery (IMR 18650 3000 mAh 3.7 V High Drain Flat Top Rechargeable Battery) was used for this study. The device was set at 3.5 V and equipped with two different coils (1.5 Ω and 0.25 Ω) to obtain a total wattage of 8±2 W and 40±5 W, respectively. These resistance values were chosen since they are considered as “safe” and “hazardous”, respectively, according to the information given to the users. The 2.5-mL Pyrex glass tank in was refilled with a nicotine-free e-liquid composed by a propylene glycol/vegetable glycerine (PG/VG) base solution (50/50, v/v) and a red fruits flavor concentrate added to a final concentration of 10% (v/v).

Determination of carbonyl compounds

Volatile carbonyl compounds were determined by headspace-solid phase micro extraction (HS-SPME) coupled to gas chromatography-mass spectrometry (GC/MS Q2010 Plus, Shimadzu, Japan). Volatile compounds were extracted from the box headspace at room temperature for 2 min, by using a triphasic SPME device (DVB/CAR/PDMS, 50/30 mm thickness, 10-mm length), which had been previously conditioned at 270 °C for 60 min. Once the volatile compounds had adsorbed onto the SPME fibre, the latter was desorbed in the GC/MS injector at 250 °C for 10 min. ARTX-WAX column (30 m x 0.25 mm i.d. x 0.25 μm film thickness, Restek, USA) was used for the chromatographic separation and the injection was carried out in the split mode, with a 1:20 split ratio. The carrier gas was helium, and its linear velocity was set at 36.2 cm/sec. The oven temperature program comprised an initial isotherm at 35 °C for 10 min, which was afterwards risen to 240 °C at 30 °C/min. The injector and interface temperatures were fixed at 250 and 230 °C, respectively. To recognize the compounds of interest, the mass spectra and retention time were compared with those of the corresponding standards. Both the acquisition and integration were performed in the single ion-monitoring (SIM) mode. Formaldehyde, acetaldehyde and acrolein were recognized and quantified by their corresponding characteristic ions (*m/z* 29, 44 and 56, respectively). As suggested in literature (Wang et al., 2017; Geiss et al., 2016), a normalized response factor (Rf) was calculated using the amount of aldehydes present in the environment as basal level, according to the following expression:

$$Rf = (A_x - A_y) / A_y$$

where A_x and A_y represent the peak areas of aldehydes detected after and before (basal) the vaping process in the exposure box, respectively.

Animal care and exposure

The EU Directive (2010/63/EU) guidelines were followed during the entire experiment. The experimental protocol was approved the Committee on the Ethics of Animal Experiments of the University of Bologna and from the Italian Ministry of Health (Permit number 26832015). The Animal Welfare Committee monitored the proceedings to ensure that all efforts have been made to minimize animal suffering. Thirty male Sprague Dawley rats (ENVIGO RMS S.r.l., San Pietro al Natisone, Udine, Italy), 7 weeks old, were housed under standard conditions (12 h light-dark cycle, 22 °C, 60% humidity). Animals had continuous access to water and chow throughout the experiment. After one week of acclimatization, animals were randomly divided in three experimental units: a control group (10 rats), and two treated groups (1.5 Ω and 0.25 Ω) composed by 10 rats each. The treated groups were exposed to the vapour generated by the e-cigarettes (see section 2.2 for details on device settings) for 28 days, as previously reported by Canistro et al. (Canistro et al., 2017) with some modifications. The whole body exposure consisted of 11 cycles of two puffs (6 sec on; 5 sec off; 6 sec on), followed by 20 min of recovery. At the end of each cycle, the animals were moved to a clean chamber. Five animals were placed in each inhalation chamber, which consisted of a propylene box with a capacity of 30 L. E-cig treated animals were subjected to the procedure 3 h/day. The levels of O₂, N₂ and CO₂ were monitored by GC/MS to establish safe O₂/N₂ and CO₂/O₂ ratios.

Tissue collection

After 24 h from the last exposure, blood was collected from the tail vein. Samples were stored in K2 EDTA tubes at 4 °C until DNA unwinding assay.

Animals were anesthetized with Zoletil 100 (100 mg/kg b.w.) and sacrificed by decapitation according to the Italian Ministerial guidelines for the species. Lung was removed, immediately frozen into liquid nitrogen, and stored at -80 °C. Lung was homogenized in 150 nM NaCl, 1 mM EDTA, 1% Triton-X, and 20 mM TRIS-HCl pH 7.4, by using a IKA Ultra-Turrax homogenizer. The homogenate was then centrifuged at 9,000 g for 15 min at 4 °C and the supernatant was collected, which from now on will be labelled as S9 fraction. The cytosolic and microsomal fractions were obtained according to previously reported procedures (Bonamassa et al., 2016).

Protein concentration

Protein concentration was determined according to the method described by Lowry et al. (1951), using bovine serum albumin as standard. Samples were properly diluted in order to reach a suitable protein concentration (Canistro et al., 2012).

Antioxidant enzymes

The following assays were performed on cytosol subcellular fraction from lung tissue.

Superoxide dismutase activity (SOD). The enzymatic activity was assayed spectrophotometrically at 320 nm by monitoring the generation of adrenochrome, one of the main products of epinephrine autoxidation at pH 10.2. The dejection of autoxidation was used to calculate SOD activity using the extinction coefficient of 4.02 per $\text{mM} \times \text{cm}$, and expressed as mol of epinephrine oxidized/min per mg protein, derived by subtracting each test curve from the epinephrine autoxidation standard curve. Activity is expressed in $\text{nmol mg}^{-1}\text{min}^{-1}$.

NAD(P)H:quinone reductase (NQO1) from lung tissue. NQO1 activity was assayed spectrophotometrically at 600 nm by monitoring the reduction of the blue redox dye of DCPIP ($\epsilon = 9.6 \text{ mM}^{-1} \text{ cm}^{-1}$), and expressed as mol of DCPIP reduced per minute per mg protein. Activity is expressed in $\text{nmol mg}^{-1}\text{min}^{-1}$.

Oxidized glutathione reductase activity (GSSG-red) from lung tissue. 1.5 mM NADPH was added to 50 mM potassium phosphate buffer, 1 mM EDTA, cytosol sample and 20mM GSSG. The generation of NADP⁺ from NADPH due to the reduction of GSSG was recorded at 340 nm for 5 min at 37°C. GSSG-red activity was calculated using the extinction coefficient of 6.22 per $\text{mM} \times \text{cm}$, and expressed as mol of NADPH consumed/min per mg protein. Activity is expressed in $\text{nmol mg}^{-1}\text{min}^{-1}$.

GSH-Peroxidase (GSH-Px) from lung tissue. The enzymatic activity was determined following the NADPH consumption at 340 nm for 5 min at 37 °C and expressed as nmol of NADPH consumed per min per mg of protein. All details have been previously reported (Melega et al., 2013). Activity is expressed in $\text{nmol mg}^{-1}\text{min}^{-1}$.

Catalase (CAT). 30 mM H₂O₂ was added to the reaction mixture, constituted by 50 mM potassium phosphate buffer and cytosol sample. The decomposition of the substrate was measured at 240 nm and catalase activity was expressed as mol of H₂O₂ consumed per minute per mg protein using a molar extinction coefficient of 43.6 $\text{mM}^{-1} \text{ cm}^{-1}$. Activity is expressed in $\mu\text{mol mg}^{-1}\text{min}^{-1}$.

Xenobiotic phase-I enzymes

The following assays were performed on microsomal subcellular fraction from lung tissue and was previously described in detail by Cirillo et al. (2016).

Pentoxoresorufin O-dealkylase (PROD) activity-CYP2B1/2, methoxyresorufin O-demethylase (MROD)-CYP1A2 and ethoxyresorufin O-deethylase, (EROD)-CYP1A1. For the reaction mixture (PROD, MROD, EROD) 0.025 mM MgCl₂, 200 mM pentoxoresorufin, 5 mM methoxyresorufin and 1.7 mM ethoxyresorufin respectively were mixed with 0.32 mg of proteins and 130 mM NADPH in 2.0 mL 0.05 M Tris-HCl buffer (pH 7.4). Resorufin formation at 37°C was calculated by comparing the rate of increase in relative fluorescence to the fluorescence of known amounts of resorufin (excitation 563 nm, emission 586 nm). Activity is expressed in pmol mg⁻¹min⁻¹.

Aminopyrine N-demethylase (APND) activity-CYP3A1/2. A total incubation volume of 3 mL, composed of 0.5 ml water solution of 50 mM aminopyrine, 25 mM MgCl₂, 1.48 ml of 0.60 mM NADP⁺, 3.33 mM G6P in 50 mM Tris-HCl buffer (pH 7.4), 0.02 mL G6PDH (0.93 U/ml) and 0.125 ml of sample was incubated for 5 min at 37°C. The reaction of the released of CH₂O with the Nash reagent generated a yellow colour that was read at 412 nm, and the molar absorptivity of 8,000 used for calculation. Activity is expressed in nmol mg⁻¹min⁻¹.

p-nitrophenol hydroxylase (p-NPH) activity-CYP2E1. 2 mM p-nitrophenol in 50 mM Tris-HCl buffer (pH 7.4), 5 mM MgCl₂, and a NADPH-generating system consisting of 0.4 mM NADP⁺, 30 mM isocytate, 0.2 U of isocytate dehydrogenase and 1.5 mg of proteins were mixed in a total volume of 2 mL. After 10 min of incubation at 37°C, 0.5 ml of 0.6 N perchloric acid was added to develop the reaction. Precipitated proteins were removed by centrifugation and 1 mL of the resultant supernatant was mixed with 1 ml of 10 N NaOH. Absorbance at 546 nm was immediately recorded and 4-nitrocathecol determined ($\epsilon = 10.28 \text{ mM}^{-1} \text{ cm}^{-1}$). Activity is expressed in nmol mg⁻¹min⁻¹.

Xenobiotic phase-II enzymes

Glutathione S-transferase (GST) (Sapone et al., 2016). The incubation mixture consisted of 1 mM glutathione + 1 mM 1-chloro-2,4-dinitrobenzene (CDNB) in methanol + 0.025 mL of sample (cytosol subcellular fraction from lung tissue) in a final volume of 2.5 mL 0.1 M phosphate Na⁺/K⁺ + buffer (pH 6.5). The product of the reaction was read at 340 nm ($\epsilon = 9.6 \text{ mM}^{-1} \text{ cm}^{-1}$). Activity is expressed in nmol mg⁻¹min⁻¹.

UDP-glucuronosyl transferase (UDP-GT) (Vivarelli et al., 2016). The activity in microsomal subcellular fraction from lung tissue was determined kinetically using 1-naphtol as substrate (final concentration, 50 mM) by the continuous fluorimetric (excitation 390 nm; emission 440 nm) monitoring of 1-naphtholglucuronide production in the presence of 1 mM uridine-5' -

diphosphoglucuronic acid. The sensitivity of the reaction was improved by performing the reaction in the presence or absence of Triton X-100 (0.2%) as a detergent. Activity is expressed in $\text{nmol mg}^{-1}\text{min}^{-1}$.

Xanthine oxidase (XO)

XO was spectrophotometrically measured in lung cytosol by quantifying the formation of uric acid at 290 nm. The reaction mixture contained 50 mM sodium phosphate buffer (pH 7.8), hypoxanthine (50 μM final concentration) and it was incubated at 37 °C for 5 min. The reaction started with the addition of NAD^+ (Shintani, 2013). Activity is expressed in $\text{nmol mg}^{-1}\text{min}^{-1}$.

ROS content in lung

2',7'-dichlorofluorescein diacetate (DCFH-DA) was used as a probe for the estimation of ROS content in S9 fraction. Samples were mixed with DCFH-DA (100 μM) at 37 °C for 30 min, and the reaction was then shut down by chilling (Kang et al. 2018). The formation of the oxidized break down product (2',7'-dichlorofluorescein, DCF) was monitored with a fluorescence spectrophotometer (488 nm excitation; 525 nm emission). DCF was quantified using a standard curve, as previously reported by Rodrigues Siqueira et al. (2005) and expressed as molar concentration per mg of protein ($\text{nM DCF mg}^{-1}\text{ protein}$).

Protein carbonylation

Protein carbonyl groups were measured as suggested by Levine et al. (1994); the method is based on the reaction of carbonyl groups with dinitrophenyl-hydrazine (DNPH), to form a stable hydrazine that can be spectrophotometrically monitored at 390 nm. Samples (cytosol from lung tissue) were prepared according as previously reported (Vivarelli et al., 2018). The results are expressed as $\text{nmol of carbonyl groups/mg protein}$.

FRAP assay

Ferric Reductive Antioxidant Power (FRAP) was determined in plasma and lung tissues according to the procedure reported by Benzie and Strain (1996). Briefly, FRAP reagent (900 mL) containing 10 mM 2,4,6-tripyridyl-S-triazine in 40 mM HCl, 300 mM acetate buffer (pH 3.6) and 20 mM FeCl_3 was added to 30 μL of plasma or supernatant tissue. The absorbance change (at 593 nm) between the final reading and the blank was calculated for each sample and related to the absorbance of ferric standard solutions. Result is expressed in $\text{nmol Fe (III) } 0.1 \text{ mL}^{-1} \text{ plasma}$.

Lipid hydro peroxides in red blood cell membranes

Lipid hydro peroxides in red blood cells were estimated by performing FOX assay (Jiang et al. 1992). It is based on the rapid oxidation of Fe^{2+} to Fe^{3+} under acid condition and in the presence of xylenol orange dye. The Fe^{3+} -xylenol orange compounds can be spectrophotometrically monitored at 560 nm. 160 μL of sample was mixed with 840 μL of FOX reagent. The amounts of hydroperoxides were extrapolated by the use of a hydrogen peroxide standard curve. The results are expressed as μM of $\text{H}_2\text{O}_2/\text{mg}$ of protein. Further details on the method can be found in Jiang et al. (Jiang et al. 1992). Results are expressed as $\mu\text{mol H}_2\text{O}_2 \text{ mL blood}$.

Haematological analysis

The haematological analyses were performed by the Central Laboratory of Clinical Pathology (CLINLAB) of the Department of Veterinary Medical Science (University of Bologna).

Gene expression studies

Total RNA from lung tissue was isolated using Purelink RNA mini kit (Thermo Fisher Scientific, Waltham, MA, USA), according to manufacturer's recommendation. Briefly, lung samples were homogenized in lysis buffer containing 1% β -mercaptoethanol using a homogenizer SHM1 (Stuart, Bibby Scientific LTD, Staffordshire, UK) and keeping samples on ice. Homogenized samples were added to an equal volume of 70% ethanol and mixed. The solution was passed through a filter cartridge, having a silica-based membrane that binds RNA. The filter was then washed once with Wash Buffer I and twice with Wash Buffer II (both provided by Thermo Fisher Scientific). RNA was finally eluted with RNase-free water and stored at -80°C . RNA samples were quantified using Nanoquant plate (Tecan, Männedorf, Switzerland) and i-control software (Tecan). For each sample, 400 ng of total RNA were reverse transcribed using the High Capacity cDNA Reverse Transcription kit (Thermo Fisher Scientific) with RNase inhibitor according to manufacturer's instructions. Briefly, 10 μL of each sample were added to 10 μL master mix and the mixture was subjected to the appropriate thermo cycling conditions. Finally, relative quantification was performed by real-time PCR (Bio-Rad CFX Connect, Bio-Rad, Hercules, CA, USA) using Universal Master Mix (Thermo Fisher Scientific) and Taqman gene expression assay (Thermo Fisher Scientific) for the following genes: IL1 β (Rn00580432_m1), IL6 (Rn01410330_m1), TNF α (Rn99999017_m1), CCL3 (Rn00564660_m1), CCL4 (Rn00671924_m1), CSF2 (Rn01456850_m1), ALDH3A1 (Rn00694669_m1). GAPDH (Rn99999916_s1) and actin (Rn00667869_m1) were used as endogenous controls. Each measurement was performed in triplicate and data were analysed through

the $2^{-\Delta\Delta Ct}$ methods (Livak and Schmittgen, 2001). Rats non-exposed to vapor from e-cig were considered the calibrator of the gene expression experiments.

Scanning electron microscopy (SEM)

Lung and tracheal samples from control and treated animals were dissected and immediately washed in 0.1 M phosphate buffer, to remove blood or any other contaminant. Tissues were fixed with 2.5% glutaraldehyde in 0.1M phosphate buffer pH 7.2–7.4 for 1 h, post-fixed with 1% osmium tetroxide (OsO₄) in the same buffer for 2 h and, finally, dehydrated with graded ethanol (50%–100%, 5 minutes each). Critical point dried specimens were mounted on aluminium stubs. After 10 nm gold sputter-coated samples were examined with a Philips SEM at 20 kV (Burattini et al., 2016).

Transmission electron microscopy (TEM)

Tissues were immediately washed and fixed with 2.5% glutaraldehyde in 0.1 M phosphate buffer for 1 h, post-fixed in 1% OsO₄ for 1 h, alcohol dehydrated and embedded in araldite, as reported by Salucci et al. (2017). For ultrastructural analysis thin sections were stained with uranyl acetate and lead citrate and observed with an electron microscope at 80 kV.

Statistical analyses

If not differently specified, results are expressed as mean \pm standard deviation (SD) on six independent replicates (n=6) and analysed by means of one-way ANOVA, followed by Tukey's multiple comparison test. The test was carried out at a 95% confidence level ($p \leq 0.05$), to separate means of parameters that were statistically different. For gene expression analysis, because we were interested in comparing a set of treatments against a single control mean, one-way ANOVA followed by Dunnett test was performed.

Results

Levels of formaldehyde, acetaldehyde and acrolein

Among the three monitored aldehydes, formaldehyde was the most abundant in e-cig vapors, followed by acetaldehyde and acrolein; however, the observed concentrations of carbonyls were strictly related to the resistance value. Acrolein, acetaldehyde and formaldehyde levels generated by 0.25 Ω coil was three- to seven-fold higher than those generated by the 1.50 Ω coil (**Table 1**).

Antioxidant profile and oxidative stress

A general imbalance of the antioxidant pattern of exposed animals compared to control is shown in **Figure 1**. Overall, 1.5 Ω group presented the mildest perturbations, whose magnitude became higher after exposure to e-cig vapour generated by the 0.25 Ω coil. This scenario was evident in the pulmonary glutathione reductase (GSSG-red; +156%, $p<0.01$) and catalase (CAT; -64%, $p<0.01$) (**Fig. 1a**). The suggested oxidative stress status, due to the strong induction of GSSG-red, was coupled with the opposite behaviour of conjugated phase-II glutathione S-transferases (GST) and UDP-glucuronyl-transferase (UDPGT), which is significantly reduced in 1.5 Ω exposed rats but increased in 0.25 Ω group (**Fig. 1b**).

Since the antioxidant enzymatic machinery appeared altered in exposed groups, we investigated the putative ROS sources. Among these, we found in the lung that XO was up-regulated proportionally to the total wattage of the device (**Fig. 1c**). We also hypothesized that changes in cytochrome P450 (CYP) catalytic cycle could be involved in ROS generation and pulmonary toxicity (**Fig. 1d**). We found the higher and biologically significant increase in 0.25 Ω group for both CYP 1A1 (up to 470 % compared to control, $p<0.01$) and CYP 2E1 (up to 196% vs control, $p<0.01$).

To evaluate if the boost of free radicals was involved in the pulmonary oxidative stress status (OSS), the ROS levels were measured in lung by using DCFH-DA fluorescent probe. **Fig. 1e** shows a ROS increment (about 1.5-fold) in 1.5 Ω exposed group compared to control and 2-fold increase in 0.25 Ω one. Largely used as an oxidative stress biomarker, carbonyl residues in pulmonary proteins were measured (**Fig. 1f**). A slight but statistically significant increasing trend was reported in exposed animals, with an inverse correlation between the carbonylated proteins amount and the coil resistance applied to the device. However, even if statistically significant, the biological meaning of these variations can't be considered relevant. To examine whether these phenomena affect the antioxidant power at systemic level, we measured the antioxidant capacity using the FRAP approach. Data referred to FRAP in plasma are reported in **Fig. 1g** and show how the antioxidant power was significantly reduced in rats exposed to vapour from 1.5 Ω e-cig compared to control. Animals exposed to 0.25 Ω e-cig vapour presented a non-significant perturbation if compared to both 1.5 Ω and control group. On the contrary, data referred to hydro peroxide level in erythrocyte membranes (**Fig. 1h**) show a slight but significant increment only in 0.25 Ω group.

Tissue damage

Lung and trachea from each group were analysed by using SEM (**Figure 2**). **Fig. 2a** and **2d** show lung from control rats. The typical spongy structure was well organized in alveoli and bronchioles: the bronchiole diameter is constant and the air sacs are preserved. Their number and size evidently decreased in lung from 1.5 Ω exposed rats (**Fig. 2b, e**), and the lung structure from 0.25 Ω group was

disorganized and showed large areas (*) of airflow collapse (**Fig. 2c, f**). In the same way, trachea from control group presented equilibrated proportion between ciliated cells (cc) and goblet cells (gc) (**Fig. 2g, j**). Trachea from 1.5 Ω group (**Fig. 2h, k**) showed a large area of tissue disruption. In the remaining one, the proportion between cc and gc was maintained. In 0.25 Ω condition, an altered morphology and a deeply changed organization appeared in greatest part of the tissue (**Fig. 2i, l**). The diffuse tissue loss at 0.25 Ω condition (**Fig. 2i, l**), was correlated to the presence of apoptotic (ap) and necrotic (n) cells (**Fig. 2l**).

This behaviour was also supported by TEM, visible in **Figure 3**. Control group (**Fig. 3a**) showed well-preserved airway epithelium cells. At high magnification (**Fig. 3b**) it is possible to observe cilia ultrastructural features. E-cig effects at 1.5 Ω (**Fig. 3c**) and at 0.25 Ω coils (**Fig. 3d**) showed several morphological changes, including epithelium detachment (**Fig. 3c**) and loss of cilia (**Fig. 3d**). These tissue damages were more evident in 0.25 Ω condition, also correlated to the presence of necrotic (**Fig. 3e and 3f**) and apoptotic (**Fig. 3g and 3h**) cells. The typical apoptotic cells are characterized by with chromatin condensation (**Fig. 3h**) and micronuclei (**Fig. 3g**).

Modulation of gene expression

Gene expression of pro-inflammatory cytokines such as IL1 β , IL6 and TNF α was analysed in rat lung tissue. Although no significant variation was recorded, a trend of increase in gene expression of both IL1 β and IL6 was observed after rat exposure to the vapour of e-cig in 0.25 Ω group compared to control (**Fig. 4a,b**). A decrease in the expression of TNF α was recorded (**Fig. 4c**). Besides, the expression of chemokine CCL3 and CCL4, encoding for macrophage inflammatory proteins, showed a significant decrease in 0.25 Ω group compared to control (**Fig. 4d,e**). Similarly, the colony-stimulating factor of macrophage and granulocyte colonies CSF2 showed a decrease in the lung tissue of rats exposed to 0.25 Ω vapor from e-cig (**Fig. 4f**). Finally, due to the critical role of ALDH3A1 in the oxidation of reactive aldehydes and in the cytotoxicity and genotoxicity of cigarette smoke (Jang et al., 2014), we analysed its expression in rat lung tissue exposed to e-cig vapours. No significant variation in ALDH3A1 expression was observed neither in 1.5 Ω nor in 0.25 Ω group (**Fig. 4g**).

Haematological profile

The haematocrit and haemoglobin (Hb) levels, as well as the total red blood cell (RBC) and reticulocyte (RC) count, were significantly higher in the 0.25 Ω group compared to those observed in the control (**Fig. 5a-d**). Our model evidenced non-significant changes in the 1.5 Ω group, but the variations resulted more marked when the resistance was decreased to 0.25 Ω . Lymphocytes count (**Fig 5e**) showed a dramatic drop in 0.25 Ω group but a non-significant decrease in 1.5 Ω group if

compared to control. Finally, a change in leucocytes profile is reported in **Fig. 5f**, which indicates that the number of circulating neutrophils was about 8-fold higher in 0.25 Ω group ($p < 0.01$) compared to control. The alterations of monocytes, eosinophils and basophils followed an increasing but not significant trend in 0.25 Ω group and a non-significant decreasing trend in 1.5 Ω group.

Discussion

Besides the e-liquid, e-cig users can arbitrarily adjust voltage and resistance values of the device. However, due to the thermal degradation of the e-liquid components, the inverse relationship between the electric potential difference and the resistance of the e-cig heating filament is responsible for the generation of vapours with different intensity and composition. Here, the device voltage was set at the traditional value of 3.5 V and the effects of low (0.25 Ω) and medium (1.5 Ω) resistances applied to e-cig was investigated.

We found that the amount of selected carbonyls increased as the resistance was reduced. These results are consistent with previous studies that demonstrated the influence of the e-cig total power on the aldehydes production (Geiss et al., 2016). Thus, the customization of the device can seriously influence the exposure levels to vapour-derived carcinogens. The inhalation of so elaborated vapours can compromise the antioxidant machinery and the physiological homeostasis, enhancing the susceptibility to chronic and degenerative diseases.

In our model, we found an imbalance in the enzymatic antioxidant responses. Lung GSSG-red activity was significantly higher in 0.25 Ω group compared to 1.5 Ω and control, in agreement with data referred to heavy smokers, where high levels of GSH are necessary for the detoxification process (Solak et al., 2005). Rats subjected to the vapour from the 1.5 Ω device showed a modest but significant impairment of the detoxifying enzymes, whereas, lowering down Ohms, the deeper changes reflected a general enzymatic up-regulation due to the higher levels of reactive carbonyl species.

It is known how the induction CYP superfamily can strongly contribute to ROS overproduction and, at the same time, plays a key role in the bioactivation of pre-mutagens and pre-carcinogens (Sapone et al., 2012; Vivarelli et al., 2016). In this study, various cytochrome P450 (CYP)-supported monooxygenase isoforms increased in the 0.25 Ω group compared to 1.5 Ω and control. In 0.25 Ω group, we found the strongest CYP1A1 induction, an isoform that bioactivates arylamines, dioxins, aromatic amines and polycyclic aromatic hydrocarbons (PAHs), and might culminate in DNA adducts that are known to increase lung cancer risk (Vázquez-Gómez et al., 2018). Likewise, CYP2E1 isoform, markedly boosted in our model, catalyses the metabolism of a wide variety of xenobiotics, including glycerol, acetaldehyde, aromatic compounds and nitrosamines (Cederbaum, 2014). These

data are of particular interest considering that changes in CYP-linked monooxygenases occurred despite the use of nicotine free e-liquid.

In parallel, pulmonary xanthine oxidase (XO) levels were significantly higher in the 0.25 Ω group than in 1.5 Ω and control. Elevated XO was previously found in patients with COPD compared with control subjects (Ichinose et al., 2003), in an animal model of asthma (Sugiura et al., 1999), and in lungs of animals exposed to cigarette smoke (Kim et al., 2013). Since both XO and CYP catalytic cycle are important sources of the superoxide radical, we measured ROS content in the lung tissue. Exposed rats presented significantly higher ROS levels compared to controls, suggesting that e-cig vapours produced an oxidative stress status (OSS).. The redox imbalance at systemic level was manifested through the significant reduction of plasma antioxidant capacity (FRAP) in the exposed animals. Consistently, data from 0.25 Ω group showed higher levels of hydro peroxides in erythrocyte membranes. Lipid peroxidation induces alteration of fine structures, fluidity, and permeability and modifies low-density lipoprotein to pro-atherogenic and proinflammatory forms (Greenberg et al., 2008) and generates potentially toxic products with be mutagenic and carcinogenic activity (West et al., 2006).

Several non-P450 enzyme systems participate in aldehydes metabolism and one of the most important is the aldehyde dehydrogenase (ALDH). The ALDH3A1 isoform is often up regulated in smoker lung tissue: its enforced mRNA expression is involved in tumorigenesis and was shown to attenuate cytotoxicity and DNA damage induced by cigarette smoke in human bronchial epithelial cells (Sullivan et al., 2010; Jang et al., 2014). Here, the lack of significant variation in ALDH3A1 gene expression in the tested experimental conditions, may suggest that the aldehydes we selected in e-cig vapour, was not oxidized by the ALDH3A1 isoform (Marchitti et al., 2008). Moreover, the lack of ALDH3A1 overexpression let us assume that the protection from the genotoxicity of e-cig was deficient (Canistro et al., 2017).

The OSS level, the impairment of the antioxidant machinery and the macromolecule damages we found, reflected in the morphological alterations at airway level. A loss of the typical organization in bronchioles and alveoli was evident in the 1.5 Ω group, and more conspicuous in the 0.25 Ω one. In particular, e-cig exposed animals reported alveolar destruction and bronchial epithelium disorganization. A demarcation line between a well-organized tissue and a loss of structure area was also revealed in trachea form from the 1.5 Ω group. More evidently, in 0.25 Ω rats, the number of ciliated cells was dramatically reduced and both apoptotic and necrotic cells were present. These alterations are similar to those reported by smokers and COPD patients (Macnee, 2009), and it was recently shown how e-cig aerosol exposure is associated with inflammation along with the loss of epithelial barrier function in lung cells (Gerloff et al., 2017). To further investigate the inflammatory

response to e-cig exposure in our experimental settings, we measured a panel of cytokine and chemokine gene expression: only two genes (*i.e.* CCL3 and CCL4) were significantly changed compared to controls and they were all down-regulated. The increasing trend of IL1 β and IL6 expression in 0.25 Ω group suggests that the more powerful setting may induced a stronger inflammatory status. Widely recognized as modulator of innate immune defences, IL1 β was often found enhanced in COPD patients and may played a prominent role in its pathophysiology (Botelho et al., 2011), while the release of IL6 from cells exposed to e-cig vapour occurred in a dose-dependent manner in response to the aerosol exposures (Lerner et al., 2015). Smoking impacts both innate and adaptive immunity (Qui et al., 2017), usually decreasing interferon- γ and TNF α (Strzelak et al. 2018). In our model, we recorded a similar trend for TNF in 0.25 Ω group, supporting the hypothesis that also e-cig vapours may induced an immune response. Furthermore, the expression of CCL3, CCL4 and CSF2 is in line with the reduced expression of TNF α . Even though these data are in contrast with the increased number of white blood cells in 0.25 Ω group, they could be explained in light of the findings by Meuronen and colleagues, that suggested how the inflammatory cells are incapable of producing chemokine mRNA in the lower airways in smokers (Meuronen et al., 2008). Of note, we could not analyse the protein expression of the significantly dysregulated genes. In this way, further studies need to be carried out to confirm the expression level of studied gene at protein level. As the influence of OSS and inflammatory processes on the haematological parameters in smokers has been found (Strzelak et al., 2018), we investigated the putative changes of haematocrit, Hb levels, RBC and RC count, and we observed that they were significantly higher in the 0.25 Ω group than in control. These data are in agreement with the haematological profile of patients with a smoking history (Ugbebor et al., 2011; Anandha Lakshimi et al., 2014; Kalahasthi and Berman, 2016; Malenica et al., 2017). Interestingly, as haematological alterations enhanced with the smoking intensity (Whitehead et al., 1995; Anandha Lakshimi et al., 2014), our model showed the most marked changes in the 0.25 Ω group. These observations concur in our hypothesis that vaping at low resistances leads to a more intense exposure. Existing data concerning the influence of smoking on lymphocyte profile are affected by numerous confounding factors of subpopulations (Stämpfli and Anderson, 2009; Andreoli et al., 2015). However, the drop in lymphocyte count from 1.5 Ω to 0.25 Ω group is in line with the inversely relation between the lymphocyte count in smokers and the increasing number of tobacco cigarette per day revealed by Sherke et al. (2016). Noteworthy, similar results were also obtained in a cross-sectional study conducted on children with the history of indoor exposure to tobacco smoke (El-Hodhod et al., 2010). Changes in leukocyte profile are in accordance with those emerged from clinical trials showing an increment of the neutrophils moving from light- to heavy-smokers, as well as in patients with COPD (Anandha Lakshimi et al., 2014; Jaroenpool et al., 2016).

In particular, the high number of circulating neutrophils herein recorded in the 0.25 Ω group, has been previously observed in smokers (Calapai et al., 2009; Andreoli et al., 2015).

In conclusion, the customization of the vaping experience results in a plethora of “personalized toxicological effects”, whose repercussions on health is unpredictable. Our study shows how two identically settled e-cigs (battery output, temperature and atomizer setting), loaded with the same liquid (PG/VG ratio, nicotine concentration and flavours), could generate different amounts of toxic aldehydes, as a mere effect of the value of the heating element.

We therefore suggest e-cig consumers to be cautious assuming that low-voltages may be synonymous of “safer” devices (Thomson and Lewis, 2015). In light of the findings here presented and until robust evidence from epidemiological studies on the putative public health repercussions is provided, the promotion of e-cig by scientific and public health agencies as smoking cessation aid should be considered with extreme caution.

Abbreviations

ROS, reactive oxygen species; e-cig, electronic cigarette; CDC, Centre for Disease Control and Prevention; FDA, Food and Drug Administration; COPD, chronic obstructive pulmonary diseases; VG, vegetable glycerol; PG, propylene glycol; IARC, International Agency for Research on Cancer; U.S. EPA, United States Environmental Protection Agency; GC/MS, gas chromatography-mass spectrometry; SIM, single ion-monitoring; Rf, response factor; XO, xanthine oxidase; DCFH-DA, 2',7'-dichlorofluorescein diacetate; DCF, 2',7'-dichlorofluorescein; DNPH, dinitrophenyl-hydrazine; FRAP, Ferric Reduced Antioxidant Power; SEM, scanning electron microscopy; TEM, transmission electron microscopy; GSSG-red, glutathione reductase; CAT, catalase; GST, glutathione S-transferase; UDPGT, UDP-glucuronyl-transferase; CYP, cytochrome P450; Hb, haemoglobin; RBC, red blood cell; OSS, oxidative stress status.

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Competing interests

The authors have declared that no competing interests exist.

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Figure legends

Table 1. Effects of resistance value (0.25 Ω and 1.5 Ω) on formaldehyde, acetaldehyde and acrolein levels in vapours released by e-cig.

All data are reported as mean \pm standard deviation of two independent replicates of aldehydes magnitude compared to their environment level.

Figure 1. Pro-oxidative effects of vapours generated from e-cig equipped with 1.5 Ω or 0.25 Ω coils.

(a) Pulmonary antioxidant enzymatic activity, (b) Phase II enzymatic activity in lung, (c) Xanthine oxidase activity in lung, (d) Enzymatic activity of CYP450 isoforms in lung, (e) ROS levels in lung revealed by using DCFH fluorescent dye, (f) Protein carbonylation in lung, (g) FRAP in plasma, (h) Lipid peroxidation of erythrocytes.

(a, b, c, d) Data expressed as percentage variation (mean \pm SD of six independent replicates) compared to control group arbitrarily set at 100%.

(e, f, g, h) Data expressed as mean \pm SD of six independent replicates. Results were analysed by means of one-way ANOVA, followed by Tukey's multiple comparison test.

* $p < 0.05$; ** $p < 0.01$ significant results between 1.5 Ω /0.25 Ω groups and control group.

^o $p < 0.05$; ^{oo} $p < 0.01$ significant results between 0.25 Ω and 1.5 Ω group.

Figure 2. SEM morphologic alterations of lung and trachea tissue in rats exposed to the vapors generated from e-cig equipped with 1.5 Ω or 0.25 Ω coils.

First and second lines report images from lung of control group (a, d), 1.5 Ω group (b, e), and 0.25 Ω group (c, f). Third and fourth lines report images from trachea of control group (g, j) in which both ciliated cells (cc) and goblet cells (gc) are appreciable. 1.5 Ω group (h, k), and 0.25 Ω group (i, l) show large areas without epithelium. * represents areas of alveoli collapse; n = necrotic cells; ap = apoptotic cells.

Figure 3. TEM ultrastructural alterations of trachea tissue in rats exposed to the vapors generated from e-cig equipped with 1.5 Ω or 0.25 Ω coils.

First line reports images from trachea epithelium of control group (a, b). Second line described 1.5 Ω group (c), and 0.25 Ω group (d) showing a detachment (\rightarrow) of epithelium, correlated, in both condition, by the presence of necrotic (e, f) and apoptotic (g, h) cells. n = necrotic cells; ap = apoptotic cells; m = micronuclei; \blacktriangleright = marginated chromatin.

Figure 4. Effects of vapours generate from e-cigs equipped with 1.5 Ω or 0.25 Ω coils on the pulmonary inflammatory pattern.

Relative gene expression of IL1 β (a), IL6 (b), TNF α (c), CCL3 (d), CCL4 (e), CSF2 (f) and ALDH3A1 (g). GAPDH and actin were used as endogenous controls. Data are expressed as mean \pm SEM of at least four independent replicates. Results were analysed by means of one-way ANOVA followed by Dunnet test. * $p < 0.05$; ** $p < 0.01$ significant results versus control (dashed line). The gene expression analysis was conducted on 7 rats in the control group, 7 rats in the 1.5 Ω group, and 4 rats in the 0.25 Ω group.

Figure 5. Effects of vapours generate from e-cigs equipped with 1.5 Ω or 0.25 Ω coils on the haematological profile.

(a) Haematocrit; (b) Haemoglobin; (c) red cells; (d) reticulocytes; (e) lymphocytes; (f) white cells. Data expressed as mean \pm SD of six independent replicates. Results were analysed by means of one-way ANOVA, followed by Tukey's multiple comparison test.

* $p < 0.05$; ** $p < 0.01$ significant results between 1.5 Ω /0.25 Ω groups and control group.

^o $p < 0.05$; ^{oo} $p < 0.01$ significant results between 0.25 Ω and 1.5 Ω group.