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Impairment of testicular function in electronic cigarette (e-cig, e-cigs) exposed rats under low-voltage and nicotine-free conditions

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ABSTRACT

Despite the lack of knowledge of the effects of electronic cigarettes (e-cigarettes, e-cigs) on public health, they have been proposed as a part of smoking cessation efforts. Recently, several basic scientific studies have pointed out how e-cigs can generate carcinogens, such as e-cig liquid thermal degradation by-products, and how the exposure can lead to genomic damage through inhibiting DNA repair or disrupting the redox homeostasis. However, scientific studies have pointed out how e-cigs can generate carcinogens and their release could be avoided setting the device to a low-voltage regimen. To test this feasibility, we show the effects of e-cig vapour generated from a low-voltage device filled with a nicotine-free liquid on rat testicular functions. The chemical analysis revealed the presence of carbonyls, such as formaldehyde, acetaldehyde and acrolein. Rats exposed reported a lower relative testis weight and higher levels of lactate dehydrogenase (LDH) as tissue damage marker, along with an impairment of 3β-hydroxysteroid dehydrogenase (3β-HSD), 17β-hydroxysteroid dehydrogenase (17β-HSD) and glucose-6-phosphate dehydrogenase (G6PDH) as key enzymes in the steroidogenesis pathway. The pro-oxidative environment was confirmed by the higher amount of reactive oxygen species (ROS), the development of lipid peroxidation and protein carbonylation, as well as from the disruption of antioxidant capability. Finally, we observed a higher rate of DNA unwinding in white blood cell line and boosted lipoxygenase (LOX)-linked activity, a tumour promotion marker. Even with the device setting at weak conditions, our results if extrapolated to humans suggest that exposure to e-cig vapours might alter gonads function in male vapers.

1. Introduction

Aggressively proposed as almost risk-free efforts for smoking cessation, electronic cigarettes (e-cigarettes, e-cigs) represent a real success story in the global market. Over the past years, a raise in the number of smokers seeking to switch to e-cigs has been noticed, and more worthy, their popularity has steadily grown among youth [1]. The rapid spread of e-cig is largely due to the common belief that the combustion absence would lead to a lower health risk [2], even though it has never been evidence-based. To fill the knowledge gap among putative benefits or harms associated to e-cig exposure, the scientific community has extensively spurred on working on this subject. Results from numerous studies have shed light on the presence of toxic compounds, including tobacco-specific polycyclic aromatic hydrocarbons (PAHs), nitrosamines, formaldehyde (group 1 carcinogens, International Agency for Research on Cancer, IARC, Lyon), acetaldehyde (group 2B) [3] and acrolein, a strong irritant for the skin, eyes, and nasal passages [4].

Recently, we showed how the exposure to e-cig vapours affects the lung oxidative stress-related enzyme pathways, resulting in oxygen free radicals overproduction which in turn leads to a higher susceptibly to DNA damage [5].

The relationship between cigarette smoking and male infertility has been widely investigated, and the main hypothesis ultimately attributes to oxidative stress a key role in smokers' sterility occurrence [6].

However, to date, little is known about the effects of e-cig exposure

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on testicular functions. Recent evidence has suggested how the refill liquid *per se* can impair the gonadic activity by disrupting oxidative balance and steroidogenesis [7]. Furthermore, although e-cigarettes share basic common features such as a heating element, a liquid storage tank and a battery supply, they greatly differ in terms of component performance. The voltage adjustable devices allow the consumers to manipulate the battery power output, thus reaching higher temperatures that result in an increment of nicotine delivery as well as toxic thermal breakdown products [4]. Some evidence indeed, seems to indicate that low-voltages result in non-detectable levels of formaldehyde-releasing agents [8].

Therefore, to better understand the contribution of these variables on toxicological outcomes, the main purpose of the present study was to study the effects of e-cig vapours on testicular functional marker enzymes and on redox homeostasis in a rat model, using a low-voltage (3.5 V) adjusted device and a nicotine-free e-cig liquid.

2. Material and methods

2.1. Chemicals

Acetic acid (PubChem CID:176), bovin serum albumin (PubChem CID:16132389), dichlorophenolindophenol (PubChem CID:13726) (DCPIP), Dehydroepiandrosterone (DHEA) (PubChem CID:5881), Diacetyldichlorofluorescein (PubChem CID:104913), 2′,7′-Dichlorofluorescein (PubChem CID:64944), 2,4-Dinitrophenylhydrazine (PubChem CID:3772977), epinephrine (PubChem CID:5816), ethidium bromide (PubChem CID:14710), Folin-Ciocalteu reagent, glycerol (PubChem CID:753) from Merck., glusose 6-phosphate (PubChem CID:5958) and glucose 6-phosphate dehydrogenase from Roche Diagnostic; L-glutathione oxidized (PubChem CID:71308714), L-glutathione reduced (PubChem CID:745), malondialdehyde (PubChem CID:10964), linoleic acid (PubChem CID:5280450) methanol (PubChem CID:5958), methoxyresorufin (PubChem CID:119220), 2-mercaptoethanol (PubChem CID:1567), nicotinamide adenine dinucleotide phosphate in oxidized (PubChem CID:5886) and reduced form (PubChem CID:5886) (NADPþ and NADPH), *p*-nitrophenol (PubChem CID:980), pentoxyresorufin (PubChem CID:107683), perchloric acid (PubChem CID:24247), pyruvate (PubChem CID:107735) resorufin (PubChem CID:69462), sodium dithionite (PubChem CID:24489), sorbitol (PubChem CID:5780), 2-Thiobarbituric acid (PubChem CID:2723628), trichloracetic acid (PubChem CID:6421), Triton X-100 (PubChem CID:5590), Trizma (PubChem CID:16218782), umbelliferone (Pub-Chem CID:4412127), urea (PubChem CID:1176), xanthine (PubChem CID:1188), xylenol orange (Pub-Chem CID:73041), 1-chloro-2,4-dinitrobenzene (PubChem CID:6), 1-naphtol (PubChem CID:7005), 7 ethoxyresorufin (Pub-Chem CID:3294) from Merck.

All others chemicals were highest purity commercially available.

2.2. E-cigarette and liquid refills

The electronic cigarette (e-cig) employed for the research was a commercially available variable voltage e-cig. The voltage was settled on 3.5 Volt and the device was equipped with a 1.5 Ohm resistance. The rechargeable lithium battery (3.7 V EH IMR, 18650; 3000 mAh) supplied power to the system. The resistance was daily replaced. The tank was loaded with a nicotine-free liquid (Fumador S.r.l., Milan, Italy) composed of propylene glycol (PG) and vegetable glycerine (VG) (50/50, *v/v*) flavoured with 10% red fruit aroma (Chemofont S.r.l.).

2.3. Chemical analysis on the e-cig vapour

Volatile compounds of e-cig vapour (such as formaldehyde, acetaldehyde and acrolein) were determined by headspace-solid phase microextraction (HS-SPME) coupled to gas chromatography–mass spectrometry (GC/MS), a SPME device equipped with a fused-silica fiber (10-mm length) coated with DVB/CAR/PDMS (50/30 mm thickness) was used. After conditioning at 270 °C for 60 min, the SPME fiber was exposed to the box headspace at room temperature. After 2 min exposure, the fiber was desorbed at 250 °C for 10 min in the injector of the GC/MS system (Q2010 Plus, Shimadzu, Japan). The sample was injected into a RTX-WAX column (30 m, 0.25 mm i.d., 0.25 mm i.d., 0.25 μm film thickness, Restek, USA) in split mode (1:20 split ratio). Helium was used as carrier gas with a linear velocity of 36.2 cm/s. The oven temperature was kept at 35 °C for 10 min, then raised to 240 °C at 30 °C/min. Injector and interface temperatures were set at 250 and 230 °C, respectively. Compounds were recognized by comparing their mass spectra and retention time with those of the corresponding chemical standards. The quantification of formaldehyde, acetaldehyde and acrolein signal was carried out by Single Ion Monitoring (SIM), using 29 *m/z*, 44 *m/z* and 56 *m/z* respectively.

2.4. Animal exposure

The study was carried out in accordance with the guidelines suggested by the EU Directive (2010/63/EU). The protocol received the endorsement from 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. Fourteen male Sprague-Dawley rats (7 weeks old) were purchased by ENVIGO RMS S.r.l. and housed under standard conditions (12-h light-dark cycle, 22 °C and 60% humidity). Animals had free access to water and chow throughout the experiment. After one-week acclimatization, animals were randomly assigned to the experimental units: control (7 rats) and exposed (7 rats). The exposed group was subjected to the vapour generated from the e-cigarette (see Section 2.2 for details on device settings) for 28 days. The exposure occurred daily for 3 h and it consisted of 11 cycles of two puff (6 s on; 5 s off; 6 s off), followed by 20 min of recovery. At the end of each cycle, the animals were moved to a clean chamber. The levels of O_2, N_2 and CO_2 were monitored by GC/MS to establish safe O_2/N_2 and CO_2/O_2 ratios. Further details on the exposure chamber assessment have been previously reported [5].

2.5. Tissue collection and sub-cellular fractions

After 24 h from the last exposure window, the blood was collected from the tail vein. Samples were stored in K_2 EDTA tubes at 4 °C until DNA unwinding assay.

Animals were anesthetized with Zoletil 100 (100 mg/kg b.w.); the sacrifice was obtained by decapitation according to the Italian Ministerial guidelines for the species. Testis were removed and rapidly frozen into liquid nitrogen, then stored at −80 °C. Testis were homogenized in 50 mM 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 5000*g* for 15 min at 4 °C and the supernatant was collected, which from now on will be labelled as S5 fraction. The cytosolic and microsomal fractions were obtained according to the previously reported procedures [9].

2.6. Relative testis weight through the gonadosomatic index (GSI)

The weight of the testis was recorded at the moment of the removal; then the gonadal relative weight was estimated by the gonadosomatic index (GSI index) as reported by Ullah et al. (2018) [10] and calculated as follows: GSI = *(testis weight (g) / body weight (g)) x 100.*

2.7. Testicular androgenic enzymes activities (3β-HSD; 17β-HSD)

3β-hydroxysteroid dehydrogenase (3β-HSD) and 17β-hydroxysteroid dehydrogenase (17β-HSD) activities in cytosolic fraction were

assessed photometrically following the reduction of NAD to NADH at 399 nm at 25 °C, pH 8.9. Dehydroepiandrosterone (DHEA) or testosterone were used as substrates for 3β-HSD or 17β-HSD, respectively. All details are described in the study by Jana et al. (2006) [11].

2.8. Testicular marker enzymes

2.8.1. Sorbitol dehydrogenase (SDH)

The enzymatic activity was determined as the amount of fructose reacted per unit of time, and it was measured by recording the absorbance decrease due to NADH oxidation. The reaction was carried out in 1 mL, which contained 0.75 mL of Tris buffer (111 mM, pH 7.5), 0.05 mL of NADH (1.8 mM) and 0.1 mL of cytosol. The solutions were mixed and incubated at 25 °C for about 5 min. The reaction was started by adding 0.1 mL of 66.6 mM fructose solution [11].

2.8.2. Lactate dehydrogenase (LDH)

Cytosolic LDH was based on the interconversion of lactate and pyruvate. During pyruvate reduction, an equimolar rate of NADH is oxidized to NAD resulting in an absorbance decrement at 340 nm [12].

2.8.3. Glucose-6-phosphate dehydrogenase (G6PDH)

G6PDH was assayed according to the protocol by Deutsch (1983) [13] with some modifications. The reduction rate of NADP was followed at 340 nm. The reaction volume of 1 mL contained 0.1 mL of 3.8 mM of NADP, 0.1 mL of 63 mM $MgCl_2$, 0.1 mL of 33 mM glucose-6-phosphate and 600 mL of Tris buffer pH 7.5. The mixture was incubated at 37 °C for 5 min and the reaction was started by adding the cytosol sample.

2.9. Oxidative stress markers

All the tests described below were performed in testis homogenate or subcellular fractions from fresh tissue.

2.9.1. ROS content

2′,7′-dichlorofluorescein diacetate (DCFH-DA) was used as probe for the estimation of ROS content in tissue homogenate. Samples were mixed with DCFH-DA (100 μM) at 37 °C for 30 min and the reaction was shut down by chilling [14]. The formation of the oxidized breakdown product 2′,7′-dichlorofluorescein (DCF) was monitored by means of a fluorescence spectrophotometer (488 excitation; 525 emission). The DCF was quantified using a standard curve as previously reported by Rodrigues Siqueira et al. (2005) [15] and expressed as molar concentration per mg of protein.

2.9.2. Protein carbonyl groups (PC) assay

Cytosolic protein carbonyl groups were measured in accordance with Levine et al. (1994) [16]. The method is based on the reaction of carbonyls groups with dinitrophenyl-hydrazine (DNPH), to form a stable hydrazone that can be monitored at 390 nm. The results are expressed as nmol of carbonyl groups per mg protein.

2.9.3. Malondialdehyde (MDA) levels

The concentration of malondialdehyde (MDA) in microsomal fraction was performed according to Seljeskog et al. (2006) [17], with some modifications Briefly, 200 μL of sample was mixed with 1 mL of acetic acid (20%) then 200 μL of 8% sodium dodecyl sulphate; the pH was adjusted to 4.0 using NaOH. Thiobarbituric acid (TBA) (40 mM, 1,5 mL),0020was added. Samples were vigorously mixed and placed in boiling water bath for 60 min. After cooling, 3 mL of n-butanol was added and samples were centrifuged at 10000 × *g* for 15 min. The clear butanol fraction was used for measuring the absorbance at 532 nm. A standard curve was drawn from hydrolysed 1,1,3,3-tetramethoxypropane (TEP) dissolved in water [18].

2.9.4. Lipid hydroperoxides (LOOHs)

FOX method was used for the estimation of lipid hydroperoxides in the tissue supernatant fraction S5. The method allows to measure the lipid hydroperoxides and it is based on the rapid oxidation of Fe^{2+} to Fe^{3+} under acidic condition and in presence of xylenol orange dye. The Fe^{3+}-xylenol orange compounds can be monitored at 560 nm. 160 μL of S5 fraction per sample were mixed with 840 μL of the FOX reagent. The amounts of hydroperoxides were extrapolated by the use of a hydrogen peroxide standard curve. The results are expressed as μM of H_2O_2/mg of protein. Further details about the method can be found in Jiang et al. (1992) [19].

2.10. Antioxidant and detoxifying enzymes

Several enzyme activities were determined within cytosolic fraction, as described as follows.

2.10.1. Catalase (CAT)

The procedure was carried out as suggested by Bonamassa et al. (2016) [20]. The decomposition of the substrate was measured at 240 nm. The activity was expressed as mol of H_2O_2 per min per mg of protein.

2.10.2. NAD(P)H:quinone reductase (NQO1)

The activity was assayed spectrophotometrically as previously described [21]. The reduction of dichlorophenolindophenol (DCPIP) was followed at 600 nm ($\varepsilon = 9.6\,mM^{-1}\,cm^{-1}$), and then the enzyme activity was expressed as mol of DCPIP reduced per min per mg protein.

2.10.3. Superoxide dismutase (SOD)

SOD was determined according to the assay published by Misra and Fridovich (1972) [22], with some adaptations. Briefly, the activity was assayed spectrophotometrically at 320 nm by monitoring the formation of adenochrome as epinephrine autoxidation product at pH 10.2. The enzyme activity was determined using the extinction coefficient $\varepsilon = 4.02$ per $mM^{-1}\,cm^{-1}$, and expressed as moles of epinephrine oxidized per min per mg protein, obtained by subtracting each test curves from the epinephrine autoxidation standard curve as reported previously [23].

2.10.4. Oxidized glutathione reductase activity (GSSG-red)

GSSG-red was measured by monitoring the consumption of NADPH during the reduction of GSSG at 340 nm. The activity was calculated using the extinction coefficient of $6.22\,mM^{-1}\,cm^{-1}$ and data were expressed as moles of NADPH consumed per min per mg of protein [21].

2.10.5. Glutathione peroxidase (GSH-Px)

GSH-Px 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 [24].

2.10.6. Glutathione S-transferase (GST); UDP-glucuronosyl transferase (UDP-GT)

The incubation mixture contained 0.1 M phosphate Na^+/K^+ buffer (pH 6.5), 1 mM glutathione (GSH) and 1 mM 1-chloro-2,4-dinitrobenzene (CDNB) dissolved in methanol. Once cytosol was added, the product of the reaction of the thiol group of GSH with the electrophilic group of CDNB was followed at 340 nm ($\varepsilon = 9.6\,mM^{-1}\,cm^{-1}$). UDP-GT was determined in microsomal fractions using 1-naphtol as substrate by the fluorimetric recording of 1-naphtholglucuronide production in the presence of uridine-5′-diphosphoglucuronic acid. All details have been reported previously [25].

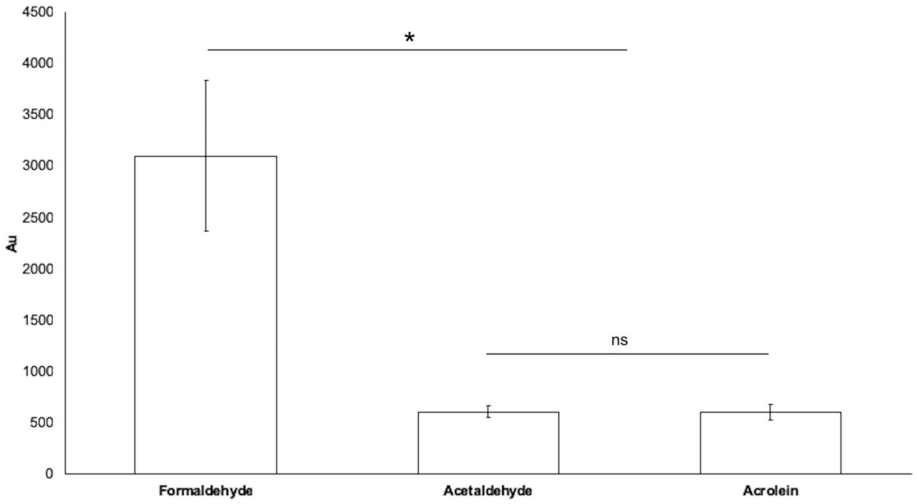

Fig. 1. Low-molecular carbonyl compounds and aldehydes detected in e-cigarette vapour.
Formaldehyde, acetaldehyde and acrolein levels detected in e-cig vapour, where formaldehyde was 5-times higher than the other two volatile compounds ($P < 0.001$). Data are reported as mean ± S.D. (expressed as peak area units) of three independent replicates ($n = 3$). Significantly different values are labelled * $P < 0.001$; ns, not significant.

2.11. Xanthine oxidase (XO)

XO was determined by quantifying the formation of uric acid spectrophotometrically at 290 nm. The reaction mixture contained 50 mM sodium phosphate buffer (pH 7.8), hypoxantine (50 μM finale concentration) and it was incubated at 37 °C for 5 min. The reaction was started with the addition of NAD^+ [26].

2.12. Alkaline DNA unwinding (FADU) test on blood

The test was assayed as previously reported by Birnoboim and Jevcak (1981) [27] with minor modifications. Three mL of blood were mixed with red blood cells (RBC) lyses buffer (10 mM TRIS-HCl, 320 mM sucrose, 5 mM magnesium chloride, 1% Triton X-100 pH 8). Samples were placed in ice for 30 min and mixed gently by inverting them many times, thus allowing cell lysis. Samples were centrifuged (0 °C, 20 min, 400 × *g*). The pellet was suspended in 2.7 mL of 0.25 M myo-inositol-10 mM sodium phosphate buffer, pH 7.2. About 0.2 mL of the last suspension was loaded in glass tubes marked as T (total ds-DNA), B (blanks) and P (partial ds-DNA) in quadruplicate. To each lysate, 0.2 mL of 9 M urea-10 mM NaOH and 0.1% sodium dodecyl sulphate (SDS) were added, and the content was incubated at 0 °C for 10 min in order to disrupt the chromatin. T samples retain natural DNA, while in B samples the DNA unwinding was induced through alkaline condition and sonication; in P samples, partial ds-DNA decomposition was achieved by the sole alkaline condition using 0.45 vol or 0.40 vol of 9 M urea-10 mM NaOH and 0.1% SDS solution in 0.2 N NaOH. Both P and B samples were kept at 15 °C for 75 min, and the denaturation was stopped by adding 0.4 mL of 1 M glucose-14 mM mercaptoethanol as neutralizing solution. T lysates were subjected to the same conditions with the only difference that the neutralizing solution was added prior to the alkaline treatment. In each tube, 3 mL of 6.7 μg ethidium bromide/mL in 133 mM NaOH solution were added and the content was mixed gently. Sample fluorescence was read at room temperature (520 nm excitation; 590 emission). The percentage of ds-DNA was calculated as (P—B)/(T-B).

2.13. Cytochrome P450 (CYP) -linked activities

p-Nitrophenol hydroxylase (pNPH; CYP2E1) was quantified by measuring 4-nitrocatechol formation at 546 nm ($\varepsilon = 10.28\ mM^{-1}\ cm^{-1}$). Pentoxyresorufin *O*-dealkylase (PROD; CYP2B1/2), ethoxyresorufin *O*-deethylase (EROD; CYP1A1) and methoxyresorufin *O*-demethylase (MROD; CYP1A2) were estimated using pentoxyresorufin, ethoxyresorufin and methoxyresorufin as substrates, respectively, and monitoring the formation of resorufin as final reaction product. The methods were described previously [28]. The activities were carried out using the microsomal fraction of the tissue.

2.14. Lipoxygenase (LOX)

Cytosolic enzyme activity was measured by following the procedure by Reddy et al. (1992) [29]. The activity was determined spectrophotometrically by recording the formation of the conjugated diene hydroperoxide at 234 nm. The reaction mixture contained 3 mL of tris-HCl buffer (50 mM, pH 6.5) and 0.1 mL of substrate (linoleic acid 3.66 mM). The enzyme activity was expressed as $\mu mol\ mg^{-1}\ min^{-1}$.

2.15. Statistical analysis

Data referred to volatile compounds are expressed as mean ± standard deviation (SD) of three independent replicates ($n = 3$) and analysed by means of one-way ANOVA to evaluate the influence of the tested conditions. One-way ANOVA, followed by Tukey's multiple comparison test, was carried out at a 95% confidence level ($P \leq 0.05$), to separate means of parameters that were statistically different. Data from analysis on testis are expressed as mean ± standard deviation (SD) resulting from at least 6 measurements from independent experiments and analysed by the non-parametric Mann-Whitney test. *P* values ≤0.05 were considered statistically significant.

3. Results

3.1. Toxic compounds released through e-cig vapour

As reported in Fig. 1, formaldehyde, acetaldehyde and acrolein were well detected in e-cig vapour; the results are reported as peak area unit. Formaldehyde was the most abundant volatile compound, whereas acetaldehyde and acrolein were 5-fold lower than formaldehyde. These results confirm that a voltage of 3.5 V and a resistance of 1.5 Ohm are able to generate toxic volatile compounds.

3.2. Anthropometric measurements

Biometric data presented in Fig. 2a show no differences in body weight gain through the experiment independently of e-cig exposure. On the contrary, focusing on testis, significant differences, albeit feeble, were observed (Fig. 2b) and they were confirmed even considering gonadosomatic index (GSI) (Fig. 2c).

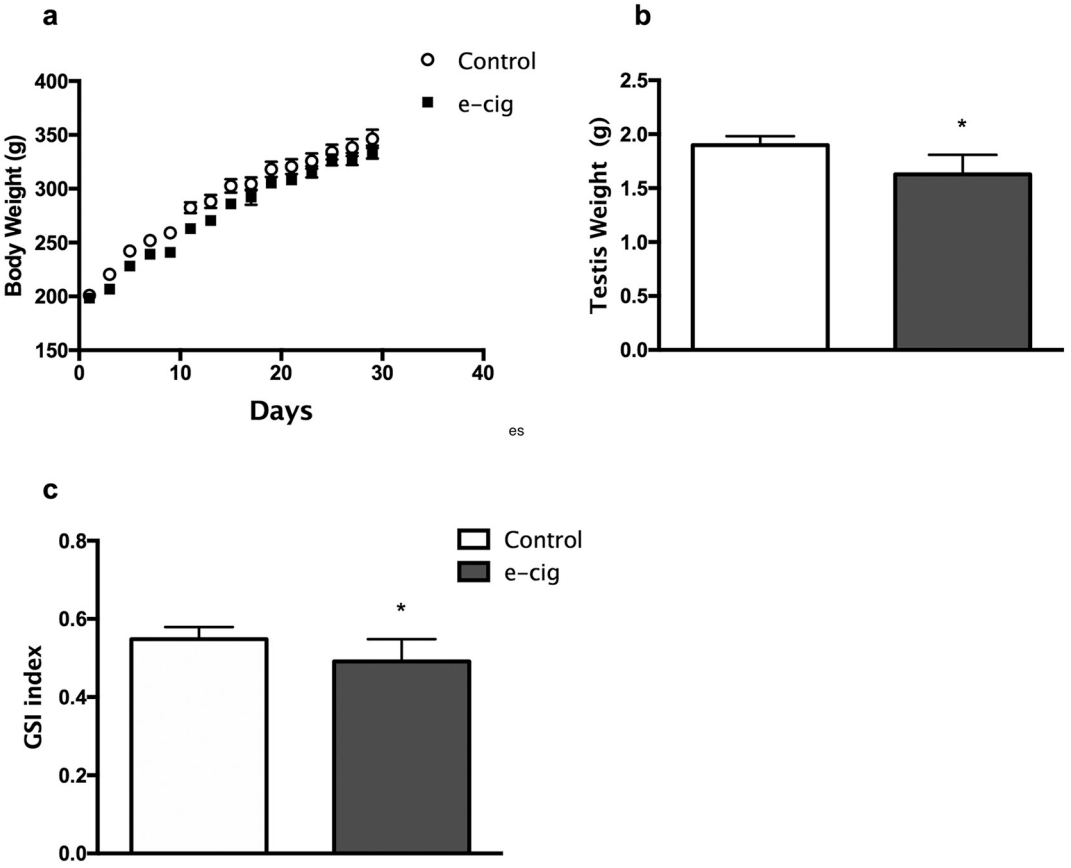

Fig. 2. E-cig vapours caused a decrease in testis weight
a. The growth curve did not show differences between exposed and control group. b. The testis weight was lower (≈ 14% loss) in exposed animals ($P < 0.05$). c. Testis weight decrement retains the statistical significance ($P < 0.05$) even adjusted for body weight trough (GSI index). The frequency distribution analysis indicated a non-Gaussian distribution of data. Results were analysed trough the non-parametric Mann-Whitney test. Data are expressed as mean ± S.D. Values significantly different from the control are labelled * $P < 0.05$, ** $P < 0.01$ ($n = 7$).

3.3. Testicular androgenic and functional marker enzymes

Fig. 3 reports data from testicular 3β-HSD and 17β-HSD as key enzymes for testosterone biosynthesis. The exposure to e-cig vapour led to a mild but significant disruption (over 20% loss; $P < 0.05$) in 3β-HSD (Fig. 3a) and 17β-HSD (Fig. 3b) catalytic activity compared to controls. SDH and G6PDH were found both significantly ($P < 0.01$) impaired in the exposed group. Conversely, LDH, marker of tissue damage, slightly raised ($P < 0.01$).

3.4. Oxidative stress markers and antioxidant-detoxifying machinery

The exposed animals reported a net increment of ROS (Fig. 4a). These data are consistent with those related to the overall oxidative tissue damage, which resulted in an increase of protein CO groups (Fig. 4b), MDA as a lipid peroxidation marker (Fig. 4c) and lipid hydroperoxides by-products (Fig. 4d). The pattern of antioxidant enzyme activities here investigated (Fig. 5a–d) showed an overall decline pattern; in particular, NQO1 and CAT reported a drastic impairment in treated animals, with decreases that overcome 60% when compared with controls ($P < 0.01$). Finally, also the increased XO (Fig. 5e), although mild, evidences a pro-oxidant framework.

On the contrary, the conjugating enzymes (Fig. 5f, g) GST and UDPGT resulted significantly ($P < 0.01$) depressed; notably, UDPGT showed a residual activity that was < 50% compared to controls. LOX, a key enzyme implicated in leukotrienes and ROS generation, as well as highly expressed tumour marker in testicular cancer, was found up-regulated in testis from treated animals ($P < 0.01$).

3.5. DNA strand breaks in white blood cells

Fig. 6 shows an increment of the percentage of unwinding DNA in white blood cells (WBC), moving from control to exposed animals. However, the test revealed no significant changes in testis tissue (data not shown).

3.6. Effects on cytochrome P450-linked monooxygenase and lipoxygenase

The e-cig vapour led to widespread induction of CYP isoforms here covered (Fig. 7a–d); in particular, the CYP2E1-linked activity resulted more than doubled in the samples from exposed animals ($P < 0.01$) (Fig. 7d). Lipoxygenase (LOX), a key enzyme implicated in leukotrienes and ROS generation, as well as highly expressed tumour marker in testicular cancer, was found up-regulated in testis from treated animals (Fig. 7e; $P < 0.01$).

4. Discussion

Extensively advertised as almost risk-free aid for smokers, the electronic cigarettes (e-cigarettes, e-cigs) have reached a massive success especially among youth and young adults [2,30]. Several toxicological outcomes associated with e-cig vapour exposure, including those related to mutagenic and co-mutagenic potential [5], changes in brain lipid homeostasis and circulating total/esterified cholesterol and triglycerides, have been recently reported [5,31], and most of them have also been independently confirmed [32,33].

As it is believed that weak conditions of use are substantial safe,

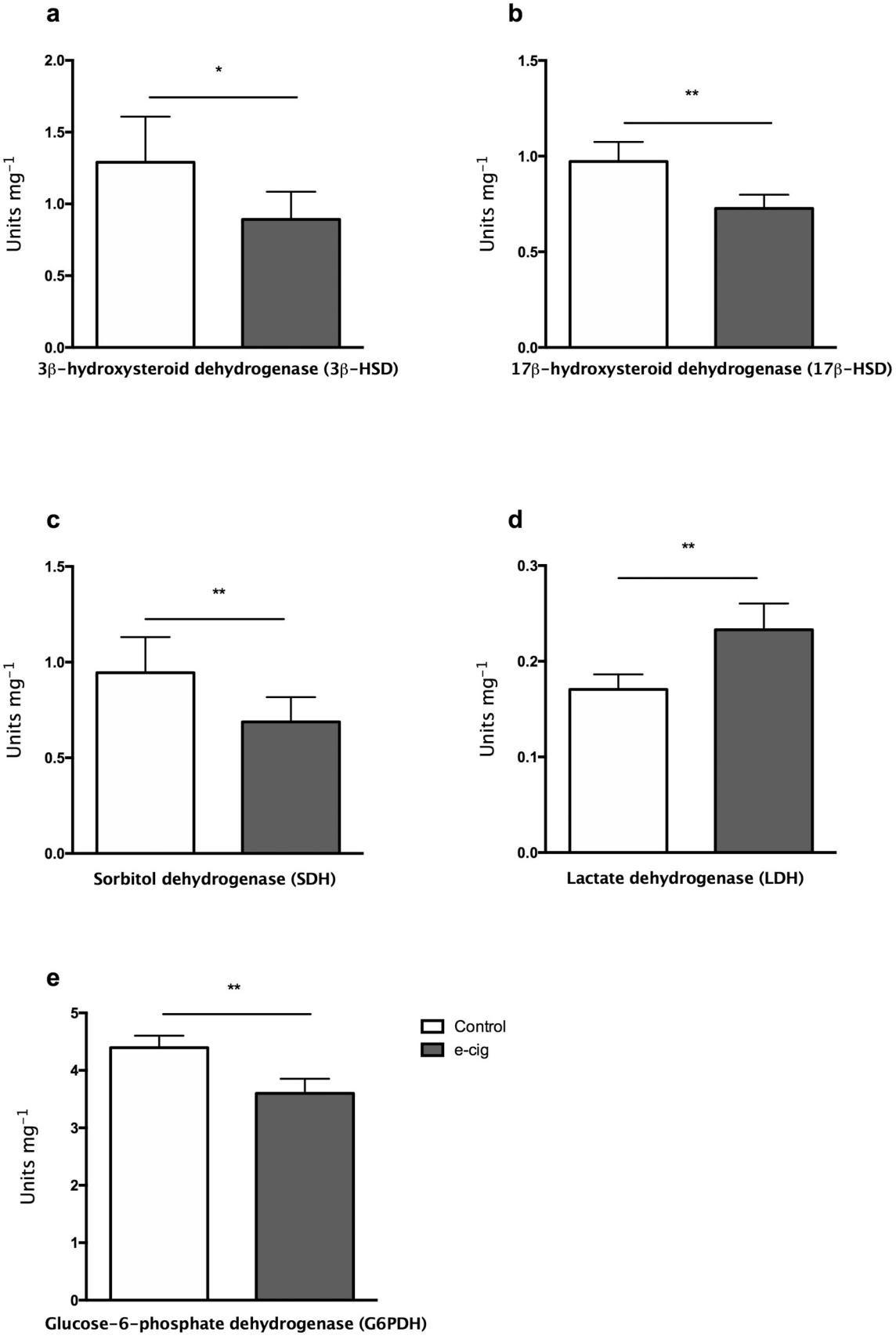

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Fig. 3. Animal exposed to e-cig vapours had an impairment of steroidogenesis enzymes and a raise of lactate dehydrogenase as a marker of tissue damage
a. E-cig vapours led to a decrement (over 25%) in 3β-HSD compared to the controls ($n = 7$; $P < 0.05$). b. The effect has also been extended to 17β-HSD ($n = 6$; $P < 0.01$) suggesting a putative impairment in testosterone synthesis pathway. c. Consistently, SDH as a testosterone dependent enzyme, resulted inhibited ($n = 6$; $P < 0.01$). d. Lactate dehydrogenase (LDH), as a marker of tissue damage, was found slightly higher (≈ 35% increase) in the exposed group ($n = 7$; $P < 0.01$). e. Glucose-6-phosphate dehydrogenase (G6PDH) plays a key role in the hydroxylation reaction during the steroid biosynthesis. The exposure to e-cig vapours resulted in a down-regulation of the enzyme capability ($n = 6$; $P < 0.01$). The frequency distribution analysis indicated a non-Gaussian distribution of data. Results were analysed trough the non-parametric Mann-Whitney test. Data are expressed as mean ± S.D. Values significantly different from the control are labelled $^{*}P < 0.05$, $^{**}P < 0.01$.

here we tested a new-generation of e-cig device, which was adjusted to one of the lowest voltages allowed (3.5 V) [34] and filled with a nicotine-free liquid having a 50/50 (*v/v*) PG/VG ratio. Since the generation of non-nicotine toxicants, such as volatile aldehydes, depends on both voltage and base-liquid composition [35], these settings were defined to avoid extreme exposure conditions; on the other hand, the lack of nicotine excludes an important confounding factor for the study of several enzymatic patterns modulation.

In the chemical analysis of the e-cig vapour, we focused on the yield of aldehydes (formaldehyde, acetaldehyde, acrolein) produced by

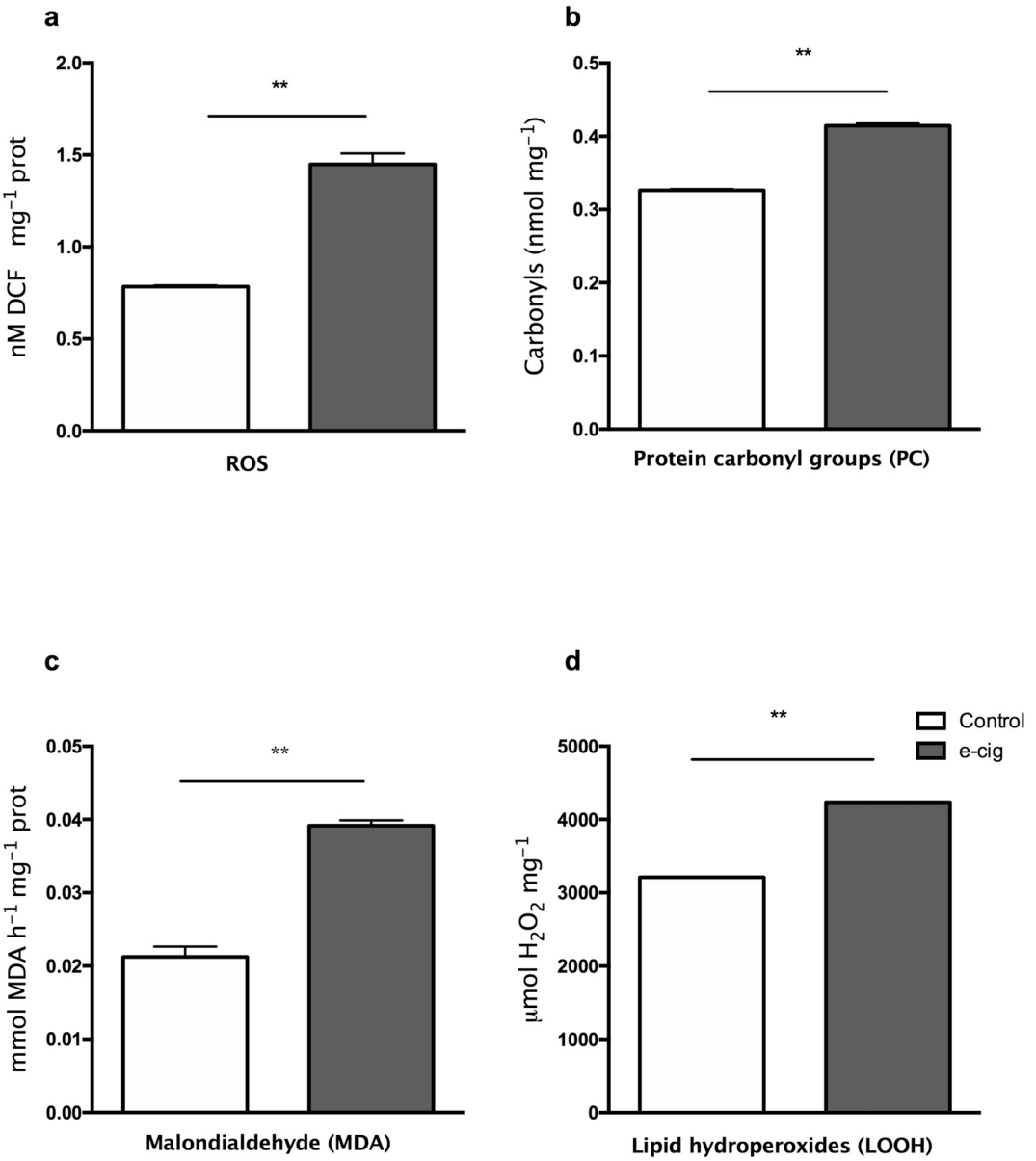

Fig. 4. Oxidative stress markers were raised in tissues from exposed animals
a. The ROS amount performed through the DFCH-DA assay showed higher levels in samples from e-cig exposed rats (> 84% of increment; $n = 6$; $P < 0.01$). b. Protein carbonyls (PC) increased slightly but significantly in testis from the treated group (≈14% increase; $n = 6$; $P < 0.01$). c. Malondialdehyde (MDA) levels here assayed as a marker of lipid peroxidation reported a marked increment ($n = 6$; $P<001$) in e-cig group *vs* control. d. Results from FOX assay to determine the lipid hydroperoxides (LOOHs) were consistent with those from other panels (≈32% increase; $n = 6$; $P < 0.01$), confirming the pro-oxidative effect of vapours from e-cig. The frequency distribution analysis indicated a non-Gaussian distribution of data. Results were analysed trough the non-parametric Mann-Whitney test. Data are expressed as mean ± S.D. Values significantly different from the control are labelled $^{*}P < 0.05$, $^{**}P < 0.01$.

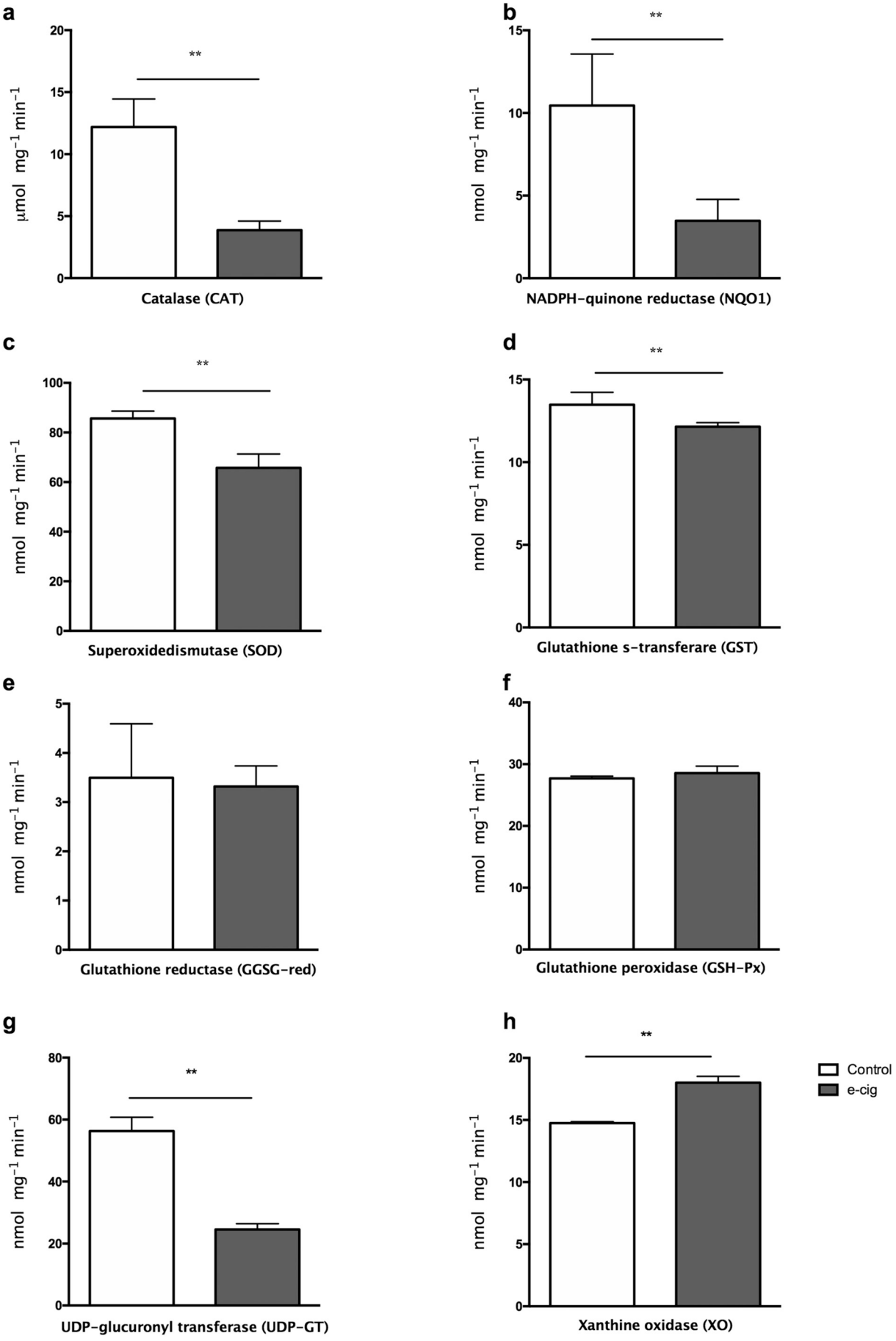

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Fig. 5. Disruptive effect of e-cig exposure on the antioxidant and detoxifying enzymatic systems
a. Catalase (CAT) activity was found markedly dropped after e-cig treatment (up to 68% loss; $n = 6$; $P < 0.01$). b. NAD(P)H:quinone reductase (NQO1) reported a fall in enzyme capability very close to that observed for CAT (up to 66% loss; $n = 6$; $P < 0.01$). c. A down-regulation was also recorded for the superoxide dismutase (SOD), although the magnitude of impairment was considerably lower (up to 23% loss; $n = 6$; $P < 0.01$). d. Glutathione peroxidase (GSH-Px) and e. Glutathione reductase (GGSG-RED) were not significantly affected by the treatment. f. The exposure to e-cig vapours resulted in a moderate inactivation of glutathione S-transferases (GST) ($\approx$ 10% loss, compared to controls; $n = 6$; $P < 0.01$), whereas, g. UDP-glucuronyl-transferase (UDP-GT) displayed a worrisome inactivation with a residual enzyme capability below the 50% of that measured in the control group ($n = 7$; $P < 0.01$). h. Samples from exposed animals showed a boosted activity of xanthine oxidase (XO) as a known ROS-generation enzyme ($n = 6$; $P < 0.01$). The frequency distribution analysis indicated a non-Gaussian distribution of data. Results were analysed trough the non-parametric Mann-Whitney test. Data are expressed as mean ± S.D. Values significantly different from the control are labelled * $P < 0.05$, ** $P < 0.01$.

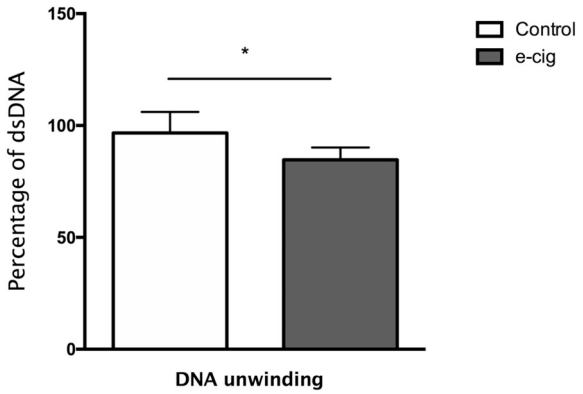

Fig. 6. Occurrence of DNA strand breaks in white blood cells from e-cig exposed rats
As it can be observed, data from exposed animals showed a mild decrement in double strand DNA percentage in blood samples from exposed animals ($n = 7$; $P < 0.05$). The frequency distribution analysis indicated a non-Gaussian distribution of data. Results were analysed trough the non-parametric Mann-Whitney test. Data are expressed as mean ± S.D. Values significantly different from the control are labelled * $P < 0.05$, ** $P < 0.01$.

thermal degradation of glycerin and propylene glycol and thus generated from almost all e-cig liquids regardless of flavourings (Sleiman et al., 2016) [36]. Our results show the presence of all screened aldehydes; of these, formaldehyde was present in the largest amount, followed by acetaldehyde and acrolein, in accordance with recent literature [36–38].

Among all the harmful effects associated with tobacco consumption, the impairment of both male and female fertility is one of the best known (Dai et al., 2015) [39]; however, the impact of e-cigs on this issue is still largely unexplored [7,40]. The presence of toxic aldehydes in e-cig vapour is of particular concern since it was recently reported how rats exposed to formaldehyde get an inhibition of sexual behaviour and the expression of steroidogenic enzymes [41,42]. Consistently with these observations, control and exposed groups exhibited similar growth curve, whereas testis weights declined in the treated subjects even when adjusted for body weights. Data from androgenic enzymes pointed to down-regulation of 3β-HSD and 17β-HSD, two key enzymes of steroidogenesis whose impairment is recognized as marker of testicular failure [10]. Interestingly, similar changes in rats injected with e-cig refill liquid have been recently found [7]. Basically, an impairment of 3β-HSD and 17β-HSD leads to a decrement of testosterone biosynthesis [43], which results in a down-regulation of testosterone-dependent enzymatic activities, such as sorbitol dehydrogenase (SDH), that decreased in exposed group. SDH plays a key role in the maturation of the germinal epithelial layer of seminiferous tubule; the increment of the marker of tissue damage LDH, together with the SDH decrease we found, suggests a deterioration of germinal epithelium [11]. We also measured the G6PDH as essential enzyme for the hydroxylation reaction during the gonadal steroid biosynthesis [44] and we observed that the exposure to e-cig vapours resulted in a down-regulation of the G6PDH capability.

A large body of literature recognizes oxidative stress status (OSS) as a leading cause of testis failure among smokers [45] and there is also plenty of evidence of the association between e-cig exposure and OSS [5,31,33,46–48]. In our study, a higher rate of ROS in the testis from exposed animals was observed, which goes along with a significant increment in protein carbonyl formation and lipid oxidation products measured as MDA and hydroperoxides amount. Further evidence addressing OSS raised from the antioxidant and detoxifying enzymatic machinery that appeared compromised as a whole; noteworthy, catalase and NQO1 showed a marked drop in catalytic activity (> 60% loss). These detrimental effects can be attributed to the release of ROS as a liquid thermal degradation by-products [49,50]; on the other hand, formaldehyde was shown to increase cellular ROS through the reaction with water-soluble radical initiators [51], as well as OSS-mediated injuries such as those above discussed including the suppression of antioxidant enzymes in male reproductive tissues [52]. Listed among ROS-generating enzymes as a well-known source of $O_2^{\cdot-}$, xantine oxidase (XO) has been also proposed as oxidative stress marker, since it is actually present in two forms: a dehydrogenase that uses NADPH as electron acceptor and an oxidase that uses the oxygen to generate H_2O_2 [53]. Oxidative stress can increase XO activity by boosting dehydrogenase-to-oxidase conversion [54]; interestingly, an increase of XO in e-cig exposed animals was here observed. At systemic level, some insights from OSS emerged from DNA damage in WBC by the alkaline unwinding FADU assay, a fluorimetric method for strand breaks detection. Our results showed a decreased percentage of ds DNA in WBC, even though data from testis did not confirm this trend (data not shown). Results are consistent with the higher levels of both MDA and ROS found, since they can chelate DNA forming highly mutagenic DNA-adducts. The oxidant/antioxidant imbalance here emerged reflects the one observed in our previous study [5].

As a whole, this data set clearly indicates that the OSS associated with e-cig vapour exposure may also occur with a low-voltage device loaded with a nicotine-free liquid, affecting organs that are not directly involved in e-cig smoking. Noteworthy, a similar picture was observed in testis from rats exposed to formaldehyde [55].

Cytochrome P450 (CYP) superfamily has a key role in the oxidation of glycerol to formaldehyde. Our results showed that e-cig exposure led to a CYP-induction of the various isoforms here tested. The increased bioactivation of ubiquitous pro-mutagens/pro-carcinogens such as polychlorinated biphenyls, aromatic amines, dioxins and PAHs by CYP1A1/2, and olefins and halogenated hydrocarbons by CYP2B1/2, can saturate the free-error repairing enzyme system, putting DNA under damage risk. The heaviest changes were recorded for *p*-nitrophenol hydroxylase (CYP2E1 – linked), showing a twice-over activity in the exposed group. This is of particular concern considering that CYP2E1 isoform is reported to be one of the most involved in the metabolism of glycerol to formaldehyde [56]; this phenomenon, coupled with that of liquid over-heating, could have contributed to formaldehyde production. Moreover, CYP2E1 activates testicular toxicants, such as benzene, acetone and styrene, turning into a higher cancer risk as well as a low sperm quality and quantity [57]. Finally, CYP induction has contributed to generate the recorded OSS due to the overproduction of ROS associated to catalytic cycle [58,59].

Several studies highlighted the link between cigarette smoke and

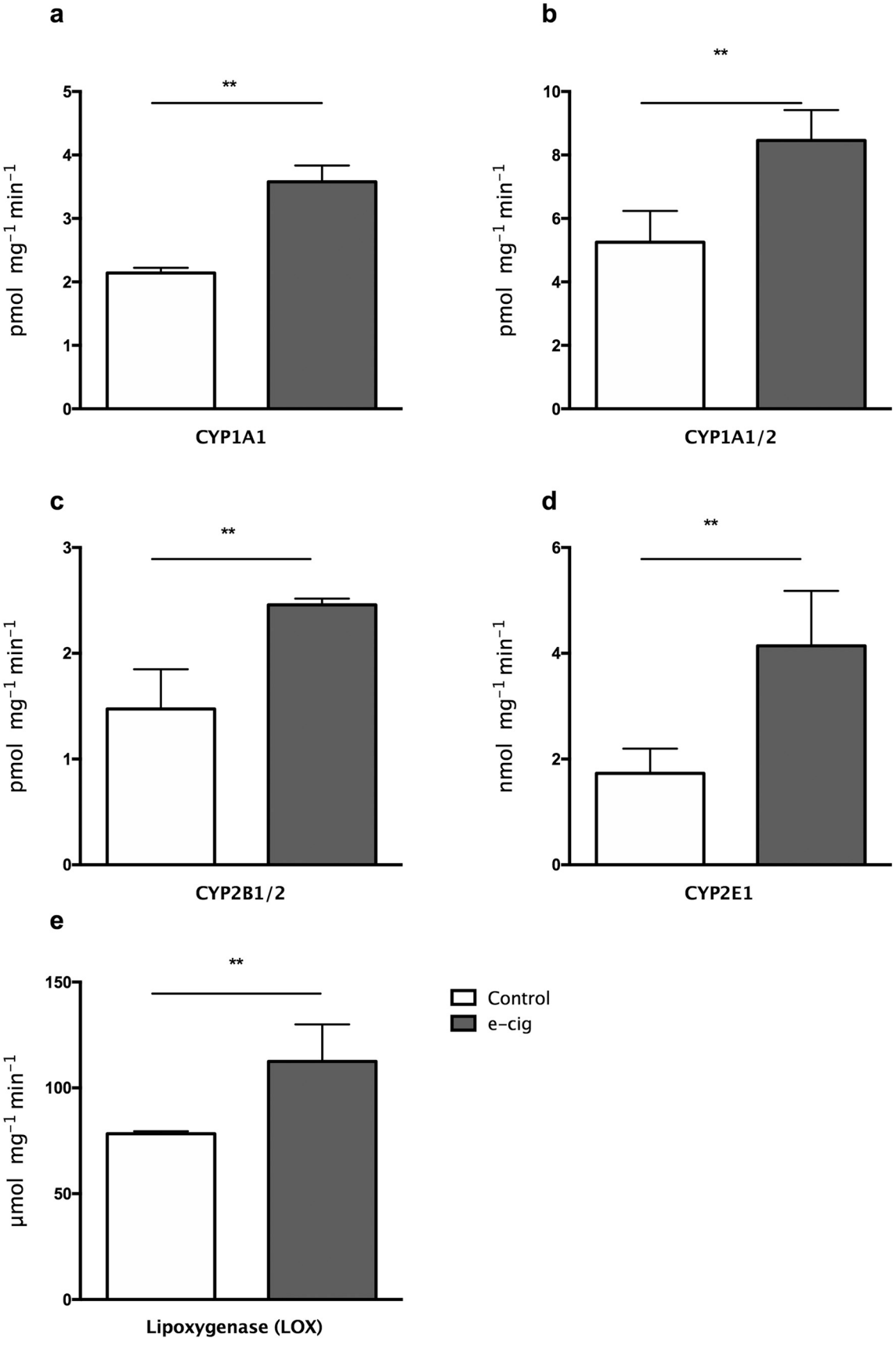

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Fig. 7. E-cig boosted cytochrome-linked monooxygenase (CYPs) and lipooxygenase (LOX)
Cytochrome P450 (CYPs) is a superfamily of major isoenzymes extensively involved in xenobiotic metabolism. In the present study the authors focused on CYPs activities since they lead to the bioactivation of ubiquitous pre-mutagens and pre-carcinogens as well as ROS release as catalytic cycle by-products. Data here presented were assayed by the use of several specific probes: a. CYP1A1 (ethoxyresorufin *O*-deethylase; EROD), b. CYP1A1/2 (methoxyresorufin *O*-demethylase; MROD), c. CYP2B1/2 (pentoxyresorufin *O*-dealkylase; PROD) were found up-regulated ranging from 60 up to 67% increase ($n = 6$; $P < 0.01$). d. The CYP2E1-linked activity was the most affected with an increment that overcomes more than twice that recorded in control group ($n = 6$; $P < 0.01$). e. Lipoxygenase (LOX) is a key enzyme implicated in leukotrienes and ROS generation as well as a tumour marker highly expressed in testicular cancer. Noteworthy, e-cig exposure led to a significant increment of LOX ($n = 6$; $P < 0.01$). The frequency distribution analysis indicated a non-Gaussian distribution of data. Results were analysed trough the non-parametric Mann-Whitney test. Data are expressed as mean ± S.D. Values significantly different from the control are labelled * $P < 0.05$, ** $P < 0.01$.

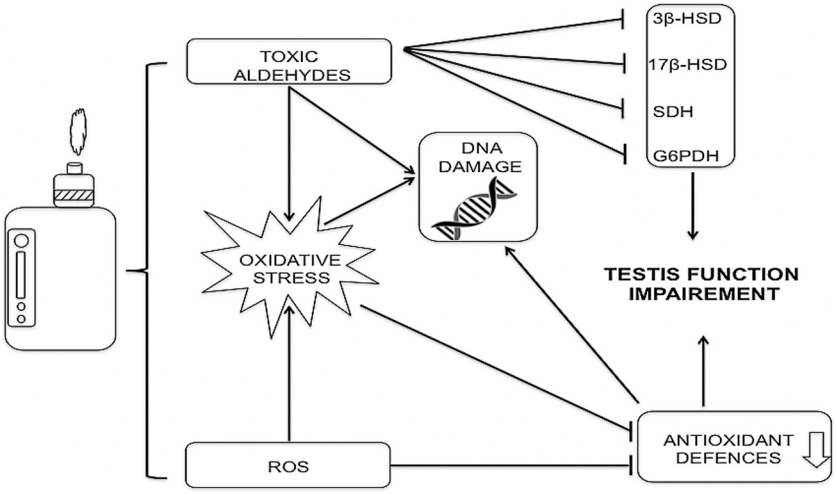

Fig. 8. Schematic representation of the hypothesized effects of e-cigarette vapour exposure on testis function impairment
The overheating of the e-liquid leads to a release of toxic aldehydes through the thermal degradation of glycerol and propylene glycol along with reactive oxygen species (ROS). Both ROS and aldehydes such as formaldehyde increase oxidative stress (OS) resulting in macromolecular damage (DNA, lipids and proteins) and contribute to the disruption of the antioxidant enzymes capability. On the other hand, aldehydes can also affect some functional markers and steroidogenic enzymes.

the up-regulation of some enzymatic pathways involved in the arachidonic acid cascade such as LOX, which is implicated in leukotrienes synthesis and ROS generation. Furthermore, this enzyme is a marker of tumour promotion, progression and metastasis, strongly expressed in testicular cancer [60]. Consistently with OSS discussed above, we observed a higher LOX-linked activity in tissues from e-cig exposed animals.

Taken as a whole, despite of using a low voltage device with a nicotine-free liquid, in the gonads the e-cig exposure disrupts enzymes involved in steroidogenesis as well as those linked to the activity of seminiferous epithelium, suggesting an impairment of the reproductive system.

5. Limitations of the study

This work does not include a sperm count, a serum testosterone measurement, as well as histological images acquisition. These tests could have surely given information on the toxicological aspects associated to the spermatogenesis. Further studies to investigate the role of e-cigs on gonadal function are therefore necessary.

6. Conclusions

The present study shows that the exposure to vapours from e-cig setting at weak conditions, such as low-voltage and nicotine-free liquid, induces detrimental effects not restricted to primary target organs, as exemplified by the impairment of the normal gonadic function through the manipulation of several key enzymatic pathways along with the increment of OSS (Fig. 8). Our findings call for caution in promoting e-cig as a part of smoking cessation efforts [61], as the knowledge gap of the putative benefits or harms for public health is still too wide.

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Competing interests

The authors have declared that no competing interests exist.

Author contributions

FV, DC and SC conceived the study and performed the *in vivo* experiments. VC and MTRE assayed the chemical analysis on e-cigarette vapour. DC, MTRE and MP contributed reagents/materials/analysis tools. FV, DC, SC and MP wrote the paper.

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