

Semen Parameter Alteration, Histological Changes and Role of Oxidative Stress in Adult Rat Epididymis on Exposure to Electronic Cigarette Refill Liquid

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Abstract

Electronic cigarettes (e-cigarettes) are devices intended to substitute conventional cigarettes, with the aim of being less harmful. In a previous report, we showed that intraperitoneal (i.p.) injection of e-cigarette liquid (E-liquid), with or without nicotine, induced toxicity in the testes of Wistar rats by disrupting oxidative balance and steroidogenesis. In the present work, we further evaluated the impact of e-liquid with or without nicotine on the epididymis of rats using the same procedure. Results showed that e-liquid treatments led to alteration of semen parameters, with a significant drop of at least 50% in sperm vitality, a significant increase of morphologically abnormal spermatozoa and an imbalance of redox status in comparison to the control group. A significant raise of 1.4 fold, compared to the untreated rats, in myeloperoxidase (MPO) granules after both treatments was recorded, suggesting an inflammatory state. Histopathological examination confirmed a marked reduction in sperm count in the cauda epididymis. Data of this study suggest that the pro-oxidant properties of e-liquid with or without nicotine, in addition to testicular defects, could lead to an inflammatory state in the epididymis, causing alterations in the semen parameters. These data provide additional information on the impact of e-liquid on the reproductive system.

Key Words: electronic cigarettes, epididymis, inflammation, oxidative stress, spermatogenesis

Introduction

The World Health Organization (WHO) recorded that due to cigarette smoking, there were nearly 6 million deaths in 2013 and predicted a mortality rate of 1 billion by the 21st century. Among the harmful effects of cigarette smoke on human health, tobacco

and nicotine induce disturbances of male reproductive functions. In particular, nicotine reduces semen quality including sperm concentration, mobility, viability and morphology (28). Nicotine also acts on the endocrine system by decreasing testosterone and hypothalamic-pituitary hormones levels (46). Furthermore, nicotine alters sperm meiotic cycle

and induces DNA impairment and chromosomal damages (49). Finally, nicotine generates oxidative stress by producing reactive oxygen species (ROS) (33, 43), which lead to serious male reproductive hazards (1, 2).

As a substitute to conventional cigarette, electronic cigarette (e-cigarette) is becoming more common in current use (35). The principle of e-cigarette is smoking cessation by getting the same smoking sensation (27) and by allowing the same gesture (24) as for the conventional cigarette. E-cigarette produces a vapor which can be flavored with light tobacco flavor, brown, fruit and the like, and may or may not contain nicotine (38).

Unlike the smoke produced by the combustion of tobacco, e-cigarette vapor does not contain tars. Furthermore, e-cigarette avoids the inhalation of more than 4,000 chemicals substances found in the smoke of classic cigarette, among which 60 are classified as carcinogenic the by International Agency for Research on Cancer (23). However, the adverse effects of e-cigarette remain poorly evaluated. It was recently shown that e-cigarette liquid (e-liquid) aerosols exhibit oxidant reactivity by generating ROS, acting like nicotine (26, 37). We have previously assessed the impact of e-cigarette refill liquid *per se* on rat liver (10), kidneys (11) and testis (12) using intraperitoneal (i.p.) injection. I.p. administration, which is largely used to assess nicotine toxicity in rodents (19, 40-42, 50), allows efficient delivery of the e-liquid directly to the testis and the epididymes, decreasing metabolism in other pathways.

In the testis, we previously identified a disrupting oxidative balance and in steroidogenesis, leading to a testicular dysfunction (12). Hence, in order to show new insights on the toxicity induced by e-liquid, we explored the role of e-liquid in the epididymis in this work by evaluating semen parameters including morphological analysis, histological changes, oxidative stress and inflammation.

Material and Methods

Chemicals

E-cigarette refill bottles certified ISO 9001, with tobacco flavor and with 18 mg/ml of nicotine or without nicotine were used. E-cigarette refill liquid is composed of propylene glycol (50%), glycerol (40%), distilled water (5-10%), flavors (1-5%) and nicotine (0-1.8%). In our previous report (13), the composition of the e-liquids was analyzed by gas chromatography-mass spectrometry (GC-MS). GC-MS analysis of the main components, which are propylene glycol and glycerol, showed that the composition of the two e-liquids differed by the presence of nicotine in the 18 mg/ml

sample. A peak corresponding to the flavoring agent diacetyl was a part of the e-liquid without nicotine.

Animal Selection and Care

Male Wistar rats weighing 160 ± 20 g were purchased from SIPHAT (Tunis, Tunisia). Before beginning the experiments, all animals were acclimated for 1 week under well-controlled conditions of temperature ($22 \pm 2^\circ\text{C}$), relative humidity ($70 \pm 4\%$), and a 12/12 h light-dark cycle with 07:30-19:30 being the light phase. Animals were housed two per polypropylene cage. They were fed with standard pellet diet (SISCO, Sfax, Tunisia) and given free access to water *ad libitum* in the duration of the experiment. Procedures involving the animals and their care followed the Guidelines for Ethical Control and Supervision in the Care and Use of Animals.

Experimental Groups

A total of 24 rats were randomized into 3 groups of 8 animals each as follow:

Control group: Each animal in this group was given i.p. injection of NaCl in a 9 g/l concentration, and in a total injection volume of 500 μl for 28 successive days.

E-liquid with nicotine (E-liquid): Each rat in this group was i.p. injected with e-liquid with nicotine (0.5 mg/kg bodyweight (BW)/day) diluted in 0.9% NaCl in a total injection volume of 500 μl for 28 successive days. E-liquid without nicotine (E-liquid 0%): Each animal in this group was i.p. injected with E-liquid 0% as for the E-liquid rats above. Since e-cigarette refill liquid is composed of propylene glycol (50%), glycerol (40%), distilled water (5-10%) and flavors (1-5%), the amount injected corresponded to, per kg BW, an average of 0.25 mg propylene glycol, 0.2 mg glycerine and 5 μg diacetyl.

Blood and Tissue Sampling

At the end of the 4-week treatment, rats were rapidly sacrificed by decapitation. Arteriovenous blood was quickly collected in heparin tubes and centrifuged at 1,000 g for 10 min at 4°C . Plasma aliquots were stored at -80°C until use. Animals were dissected and epididymes were removed, and cleared of adherent tissues. Eight epididymes of each group were stored at -80°C until biochemical analysis; three epididymes of each group were immediately fixed in buffer formalin for histological analysis and the five others were used for sperm parameters evaluation.

Evaluation of Sperm Parameters

Sperm Count

One single cauda epididymis was placed in Roswell Park Memorial Institute (RPMI) medium in a Petri dish and then shredded using a needle in a glass cup. After 10 min incubation at ambient temperature, well dispersed sperm was recovered as the stock solution. The cauda epididymal sperm count was performed according to the method of Vega *et al.* (45). Sperm solution was diluted to 1/5th, and then counted using a Malassez cell on a light microscope. Only spermatozoa heads found on the grid were counted. Each sample was counted twice and averaged. The sperm concentration was expressed as number $\times 10^6 \text{ ml}^{-1}$.

Sperm Vitality

Sperm vitality study was assessed using the Vita Eosine kit purchased from RAL diagnostics laboratories (France, ref. 380420). The staining was performed with one drop of fresh semen into two drops of staining solution on a microscope slide. Using another slide, a smear was made and allowed to dry. Unstained (intact) and red-colored (with damaged membranes) spermatozoa were counted under the microscope using the 100x objective. Sperm viability was defined as the percentage of intact cells (14, 15). Counting was performed three times on a total of 150 spermatozoa and was averaged.

Sperm Morphology

Determination of abnormal sperm morphology was performed by the Spermoscan staining kit provided by RAL diagnostics laboratories (France, ref. 366510-0000). Morphological sperm defects were evaluated and examined on an optical microscope (100 \times objective). At least one hundred spermatozoa from different fields in each slide were examined and classified for criteria of morphological abnormalities (head, tail and tail-head) (18). Abnormal sperm cells were counted and a percentage was calculated.

Testosterone Assay

The concentration of testosterone was measured by commercial enzyme-linked immune-absorbent assays (ELISA), specific for rat, purchased from Demeditec (Germany, DEV9911). Briefly, an unknown amount of testosterone present in the plasma and a defined amount of testosterone conjugated to horseradish peroxidase compete for the binding sites of testosterone antiserum coated to the wells

of a microplate. After 1-h incubation on a shaker, the microplate was washed four times. After addition of the substrate solution, the optical density was measured, and the concentration of testosterone was calculated, which is inversely proportional to the optical density.

Determination of Inflammatory Effectors

The myeloperoxidase (MPO) kit provided by RAL diagnostics laboratories (France, ref. 380420) was used to distinguish peroxidase granulations stained in red, nuclei of leukocytes stained in blue and red cells were unstained.

Assessment of Epididymal Lipid Peroxidation (LPO)

LPO was detected by the determination of malondialdehyde (MDA) production by the method of Buege and Aust (6). The reaction took place in a warm medium acid to form a pink coloring pigment intensity of which was proportional to the MDA concentration in the sample. Absorbance of the resulting chromophore was measured at 532 nm using a UV-visible spectrophotometer (France, Beckman DU 640B) MDA levels were determined by using an extinction coefficient of $1.56.105 \text{ M}^{-1} \text{ cm}^{-1}$ for the MDA-thiobarbituric acid (TBA) complex.

Determination of Epididymal Thiol (–SH)-Groups Concentration

Total concentration of –SH groups was determined according to the Ellman method (16). Briefly, a volume of 50 μl of epididymis tissue supernatant was added to 1 ml Tris base (0.25 M)-ethylenediaminetetraacetic acid (EDTA) (20 mM) buffer, pH 8.2, and absorbance was measured at 412 nm. A first reading of the optical density (DO1) at a wavelength $\lambda = 412 \text{ nm}$ was immediately carried out. A volume of 20 μl 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB) (0.1 M) was added and incubated at room temperature for 15 min. After incubation, the absorbance of the sample was measured at the same wavelength. Results were expressed as mM.

Determination of Epididymal Superoxide Dismutase (SOD) Activity

SOD activity was determined by using the modified epinephrine assay of Misra and Fridovich (29). Epinephrine was added to the assay mixture containing the tissue supernatant. Change in the extinction coefficient was followed at 480 nm in a spectrophotometer. The unit of enzyme activity is defined as the enzyme required for 50% inhibition

Table 1. Effects of e-liquid with or without nicotine on sperm concentration, morphology and vitality in rats

Groups (n = 8)	Sperm Count (10 ⁶ /ml)	Vitality (% of live sperm)	Morphology of Sperm (%)				Testosterone (ng/ml)
			Abnormality				
			Total	Rolled Up	Angular	Absent Flagellum	
Control	42.5 ± 2.0	100.0 ± 21.6	24.0 ± 0.9	7.1 ± 0.8	6.5 ± 0.4	6.1 ± 0.7	3.95 ± 0.17
E-liquid 0%	32.3 ± 3.0 [*]	27.0 ± 4.6 ^{**, #}	43.0 ± 1.0 ^{***, ##}	9.6 ± 1.0 ^{***, ###}	15.2 ± 1.1 ^{***, ###}	4.4 ± 0.5 [#]	2.20 ± 0.23 ^{***, #}
E-liquid	38.4 ± 0.9 ^{**}	42.8 ± 5.1 [*]	30.2 ± 1.8 ^{**}	11.6 ± 1.3 [*]	8.8 ± 0.6 [*]	6.4 ± 0.5	3.05 ± 0.20 [*]

Values are means ± S.E.M. for eight rats in each group. Control, without treatment; E-liquid 0% and E-liquid, e-cigarette without or with nicotine, respectively. *, # $P < 0.05$, **, ## $P < 0.01$, and *** $P < 0.001$, *Comparison of Control with other groups; # Comparison of E-liquid 0% and E-liquid.

of auto-oxidation of epinephrine, and was expressed as unit (U) per mg protein.

Determination of Epididymal Catalase (CAT) Activity

CAT activity was assayed by measuring the initial rate of H₂O₂ disappearance at 240 nm. Briefly, the assay mixture consisted of 0.05 M phosphate buffer, pH 7.0, and 0.019 M H₂O₂ in a volume of 0.3 ml. CAT activity was calculated using the extinction coefficient of 40 mM⁻¹cm⁻¹ for H₂O₂ and the data were expressed in terms of mmol of H₂O₂ consumed per minute (U) per mg protein.

Determination of Epididymal Glutathione-S-Transferase (GST) Activity

GST activity was determined according to the method of Habig *et al.* (22). Enzyme activity is proportional to the formation of a complex between glutathione and an artificial substrate for GST, 1-chloro-2, 4-dinitrobenzene (CDNB), which may be followed, at 25°C by the increase in optical density at 340 nm. GST activity was expressed in terms of nmol of CDNB-GSH conjugate formed per minute per mg protein.

Histological Analysis

Tissue specimens were taken from the epididymes of each of the three groups of rats and were fixed in formol 10% fluid. After fixation for 48 h, tissues were dehydrated through a graded series of ethanol. Tissue specimens were cleared in xylene and embedded in paraffin. The paraffin blocks were sectioned at 5-μm thickness by a rotary microtome. The obtained tissue sections were collected on glass slides and were stained using

Haematoxylin and Eosin stains. The sections were viewed and photographed using an Olympus light microscope (Olympus BX53, Tokyo, Japan).

Statistical Analysis

All values were expressed as mean ± standard error of the mean (S.E.M.). They were statistically analyzed using a one-way analysis of variance (ANOVA) and differences with a value of $P < 0.05$ were considered as statistically significant.

Results

Evaluation of the Quality of Reproductive Performance

Semen Analysis

E-liquid exposure induced a significant decrease in the epididymal spermatozoa number (Table 1). In fact, whereas sperm number was 32.3 ± 3.0 million/ml in the E-liquid 0% rats and 38.4 ± 0.9 million/ml in the E-liquid rats, the sperm count reached 42.5 ± 2 million/ml in the control rats (Table 1). In order to estimate the viability of the sperm cells, the eosine-nigrosine staining was used allowing the distinction between white alive sperm cells and pink dead sperm cells. Results showed a significant decrease in the percentage of viability for E-liquid 0% and E-liquid rats in comparison to the control group (Table 1). We then performed a morphological study of the sperms using an optical microscope. Results pointed to a significant increase of the percentage of morphological abnormalities cells with 43.0 ± 1.0% for E-liquid 0% rats and 30.2 ± 1.8% for E-liquid rats (Table 1). The most frequent abnormality of spermatozoa was found at the level of the flagellum (Fig. 1, B-D).

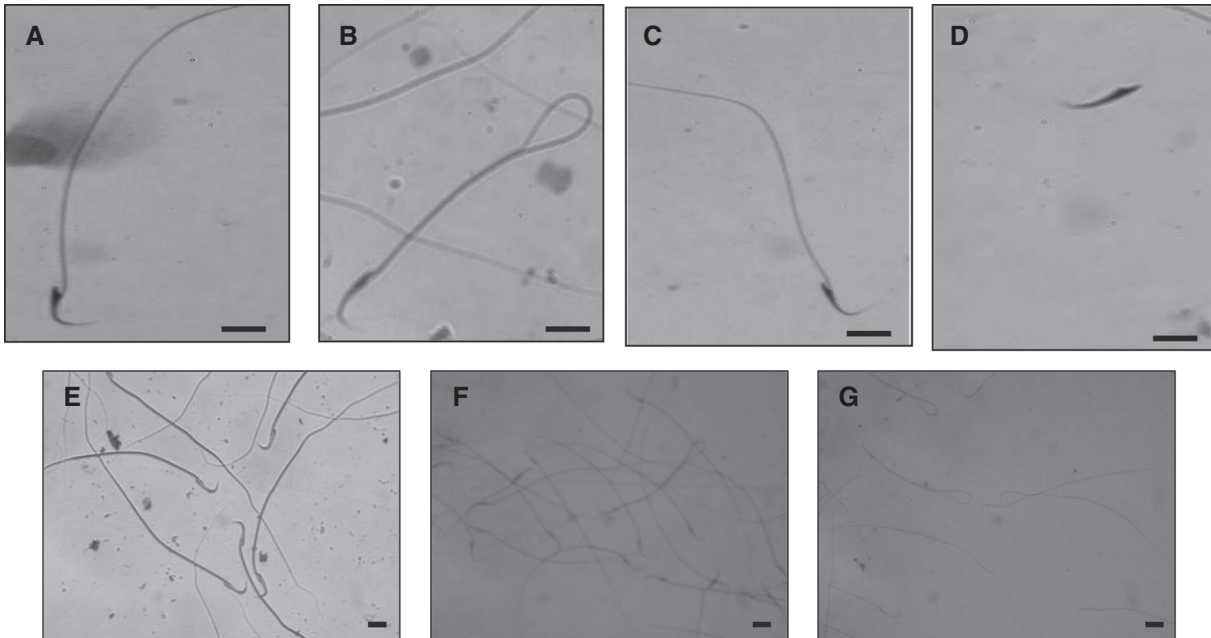


Fig. 1. Patterns of sperm abnormalities in (A) Normal spermatozoid, (B) Looped tail, (C) Flagellar angulation and (D) Absence of flagellum. Group of sperm in (E) Control, (F) E-liquid 0% and (G) E-liquid. Scale bar: 10 μ m.

Evaluation of Plasmatic Testosterone Level

After 4 weeks of e-liquid treatment, rats showed an important decrease in testosterone level in comparison to the control group with 2.20 ± 0.23 ng/ml for E-liquid 0% rats ($P < 0.001$) and 3.05 ± 0.20 ng/ml for E-liquid rats ($P < 0.05$) vs. 3.95 ± 0.17 ng/ml for the untreated rats (Table 1).

Evaluation of the Inflammatory State

After 28 days of treatment, a significant increase of the percentage of the MPO granulations was observed in the E-liquid 0% (141.98 ± 10.78 %, $P < 0.01$) and E-liquid (139.14 ± 7.70 %, $P < 0.05$) groups, in comparison to the control values (Fig. 2). Leukocyte count was also increased significantly with 176.51 ± 11.79 % in the E-liquid 0% group ($P < 0.01$) and 142.31 ± 5.59 % ($P < 0.05$) in the E-liquid group compared to the control group (Fig. 3). Percentage of erythrocytes in the spermatic fluid decreased significantly by 45.86 ± 3.21 % in the E-liquid 0% animals ($P < 0.001$) and 53.28 ± 1.37 % in the E-liquid rats ($P < 0.01$) (Fig. 4).

Oxidative Stress Status

Implication of oxidative stress was also studied (Table 2). E-liquid per se was found to induce a significant increase in the MDA level, as well as a

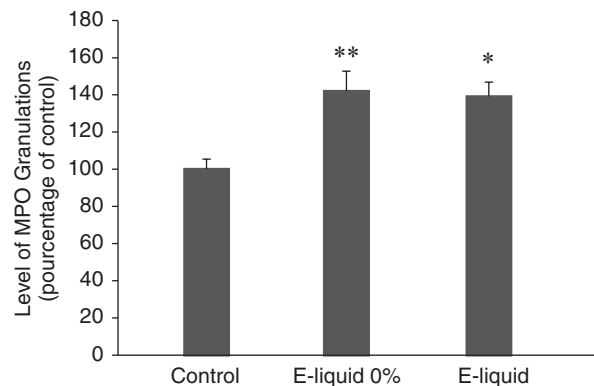


Fig. 2. Level of MPO granulations in the spermatic fluid. E-liquid 0% and E-liquid were rats treated with E-liquid without or with nicotine, respectively. * $P < 0.05$ and ** $P < 0.01$ as compared to the control group.

significantly increase of -SH groups, SOD, CAT and GST activities in the epididymis (Table 2), suggesting a moderate stress status. On the other hand, e-liquid associated to nicotine significantly reduced the CAT and GST levels (Table 2), suggesting a more intense stress installation.

Epididymis Histopathology

Epididymis sections from the control group

Table 2. Effects of e-liquids on the oxidative status of the epididymis.

	Control	E-Liquid 0%	E-Liquid
SOD (nmol/min/mg pr)	0.138 ± 0.059	0.363 ± 0.057* ^{##}	0.113 ± 0.047
CAT (U/mg pr)	0.021 ± 0.001	0.027 ± 0.001* ^{##}	0.014 ± 0.002*
GST (nmol/min/mg pr)	2.930 ± 0.371	5.520 ± 1.289*** ^{##}	0.727 ± 0.116**
SH (mM)	0.004 ± 0.007	0.012 ± 0.002* ^{##}	0.005 ± 0.001
MDA (nmol/min/mg pr)	0.229 ± 0.027	0.390 ± 0.054*	0.290 ± 0.058

See footnote to Table 1. * $P < 0.05$, **, ^{##} $P < 0.01$, and *** $P < 0.001$. *Comparison of Control with other groups; [#]Comparison of E-liquid 0% and E-liquid.

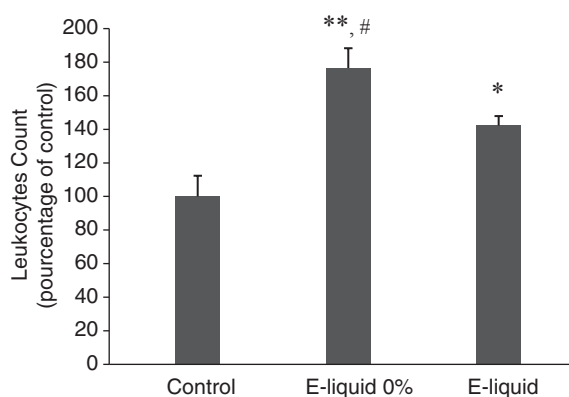


Fig. 3. Leukocytes count in the spermatic liquid. See legend to Fig. 2. [#] $P < 0.05$ as compared between the E-liquid 0% and the E-liquid groups.

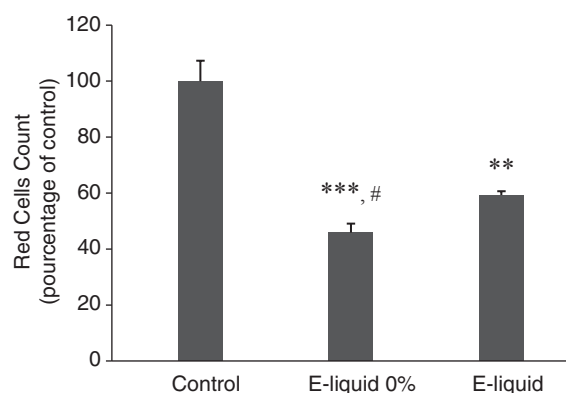


Fig. 4. Erythrocytes count in the spermatic fluid. See legend to Fig. 2. ** $P < 0.01$, and *** $P < 0.001$ as compared with the control; [#] $P < 0.05$, as compared between the E-liquid 0% and the E-liquid groups.

showed normal morphology with compactly arranged tubules. In all groups, the cauda epithelium was thick and pseudo-stratified (Fig. 5). However, while the control rats presented a high density of sperm in lumen caudal epididymis (Figs. 5A & 5B), sections of e-liquid rats showed a specific decrease of sperm density in lumen cauda epididymis, with a more pronounced reduction in rats exposed to e-liquid with nicotine (Figs. 5C to 5F).

Discussion

Many previous studies have focused on the impact of nicotine on the male reproductive system leading to alterations in both the male gonadal functions: spermatogenesis and steroidogenesis. However, there is a lack of data on e-cigarette and e-liquid exposure. We previously showed deleterious effects of e-liquid by pointing out oxidative damages, histopathological changes and steroidogenesis disturbance in the rat testis (12). In the present study,

we brought new insights about e-liquid toxicity in the rat epididymis by highlighting epididymal oxidative stress imbalance and inflammation, leading to a spermatogenesis shortage.

Data of the literature have identified that inhibition of spermatogenesis is due to nicotine and its main metabolite, cotinine (34). In fact, several studies correlated the reduction in spermatic concentration to a decrease of the testosterone level and to the interaction between nicotine and cell membranes leading to structural alterations of the male gametes. The overall data support the idea that nicotine induces an alteration in the production of sperm cells (3, 9, 32, 39). Hence, our results concerning the charging liquid with nicotine could be explained by an alteration in spermatogenesis as a result of nicotine action.

E-liquid without nicotine may also induce disruption in spermatogenesis or increases of sperm mortality by excessive apoptosis. Exposure to propylene glycol, the major component of e-liquid, in male Sprague-Dawley rats was shown to induce a

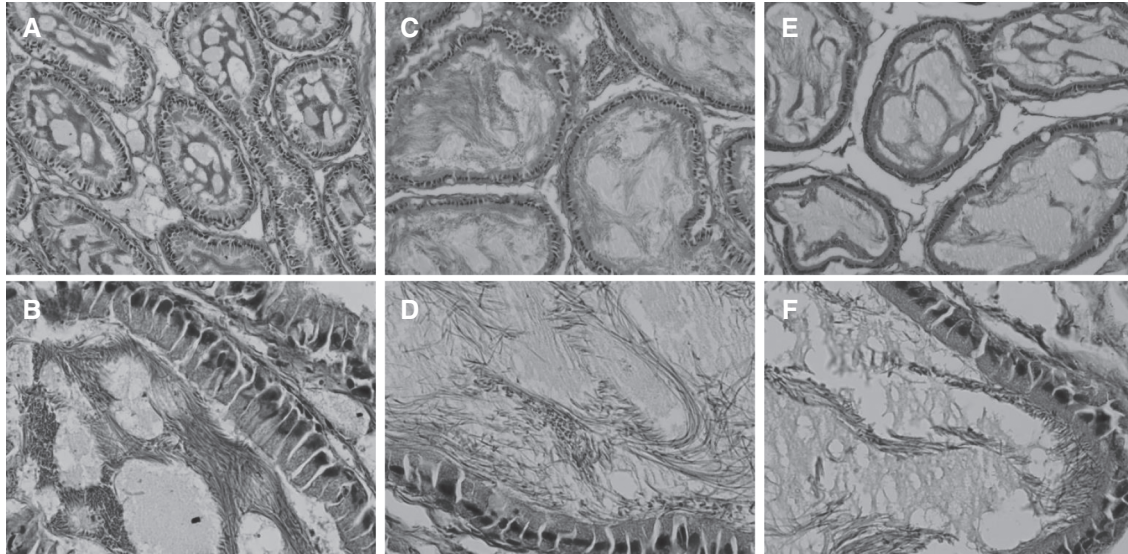


Fig. 5. Photomicrographs of cauda epididymis in control (A, B), E-liquid 0% (C, D) and E-liquid (E, F) showing decrease sperm quantity in the lumen cauda epididymis after treatment with E-liquid 0% (C, D) or E-liquid (E, F). A, C, E: x100, B, D, F: x400.

decrease in the number of sperm cells with specific damages localized in the flagellum (25). Hence, propylene glycol contained in E-liquid 0% could be responsible for the highlighted observations in our study.

Moreover, oxidative stress is described as one of the major causes of infertility (48). Indeed, in spite of the balance of pro- and antioxidant molecules in the environment of sperm cells, endogenous and exogenous factors can stimulate the ROS production and retrofit the antioxidative defense situation leading to oxidative stress. The change in antioxidant activity depends on the nature of the stress: an increase of the antioxidant capacity occurs at relatively low levels of stress when the body is able to neutralize the ROS overproduction; however, under high levels of stress, this capacity fails (41). E-liquid not associated to nicotine has been shown to significantly increase the MDA level as well as the activities of -SH groups, SOD, CAT and GST. Hence, we can conclude that E-liquid 0% induces a moderate stress level in the epididymis. These results are similar to the recent studies describing the ability of e-liquid aerosol to generate ROS in lung epithelial cells and in human gingival fibroblasts (26, 37).

E-liquid with nicotine treatment results in the decrease of antioxidants enzyme activities, like CAT and GST, indicating the installation of an important oxidative stress in the epididymis. Nicotine is known to increase ROS by breaking down the mitochondrial respiratory chain (20) and to modify the antioxidant system (5, 30). Moreover, we have previously shown that e-liquid treatment could

induce a modification of oxidative status in different organs (10, 11), especially in the testis causing steroidogenesis alterations (12). Thus, nicotine action could explain the obtained results of E-liquid group.

In the epididymis, we can conclude that E-liquid 0% or E-liquid promotes oxidative stress leading to a reduction of sperm count and a disruption of spermatogenesis. Supporting this finding, this study showed that e-liquid exposure induced histopathological changes in the epididymis, showing decreased sperm quantity in the lumen cauda epididymis after treatments with E-liquid 0% or E-liquid. Furthermore, as inflammation plays an important role in the regulation of spermatogenesis (31), the number of MPO granulations and leukocytes in the spermatic liquid was quantified. A significant increase in MPO granulations and leukocytes, associated to a significant decrease in the number of red blood cells, was identified. Although other *in vitro* studies revealed the inflammatory effects of e-cigarette vapor in skin and lung cells (7, 47), the present study is the first to show an epididymal inflammation induced by e-liquid independently of nicotine action.

Since testes and epididymes are organs rich in polyunsaturated fatty acids and have low antioxidant defense, these organs are more susceptible to tissue damages by E-liquid. Nicotine, by interacting with cellular membranes, induces structural alterations and the recruitment of neutrophils, macrophages and monocytes containing MPO (36). We postulate here that nicotine, which is a pro-inflammatory agent, could increase cyclooxygenase-2 (COX-2) expression

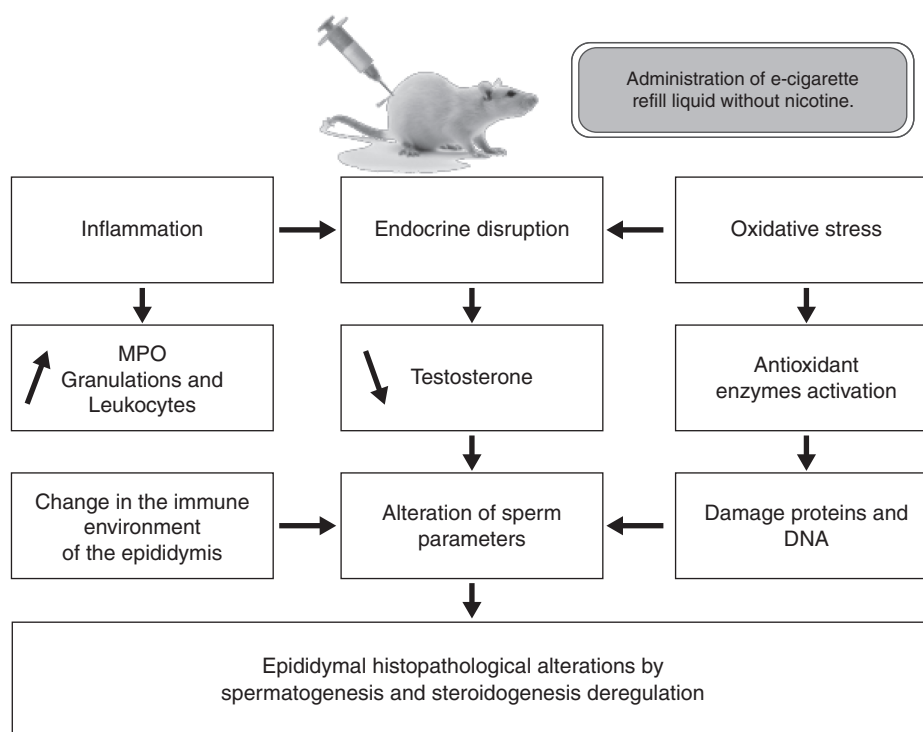


Fig. 6. Hypothetical mechanism explaining reprotoxicity after treatment with electronic refill liquid without nicotine.

and generate oxidative stress, leading to a disruption of the reproductive system (8). On the other hand, no comprehensive study has revealed inflammation in the testis and/or epididymis after exposure to E-liquid 0%. However, some authors have suggested that e-liquid may contain some endotoxins, which could inhibit innate immunity (17, 44).

To explain e-liquid toxicity in rat epididymis, a hypothetical mechanism is proposed (Fig. 6). In this mechanism, e-liquid could modify the balance of pro- and anti-oxidant by increasing the production of free radicals, which could cause damages to proteins and DNA, hence reducing the number of germ cells and modifying spermatogenesis. The e-liquid could also promote inflammation by increasing the number of leukocytes and MPO granules. This inflammation would act on spermatogenesis leading to an alteration of the spermatogenic parameters. Finally, by decreasing the levels of plasma testosterone, e-liquid could lead to further spermatogenesis modifications.

Limitations of the Study

Some limitations of our study must be considered. The major one is the use of intra-peritoneal injection of e-liquid and not by aerosol. I.p. injection allows efficient delivery to the testis and epididymis, decreasing metabolism by other pathways. As a comparison, an average e-cigarette smoker weighing

60 kg inhales a bottle of e-liquid (10 ml) with 16 mg of nicotine/ml (17), corresponding to 2.5 mg nicotine/kg BW/day. In our study, we injected 0.5 mg nicotine/kg BW/day into the rats, *i.e.* using a lower dose but in a single injection. Moreover, inhaled e-liquids are heated, an act which transforms the components of the e-liquid and increases their toxicity by raising the content in free radicals (44). Hence, the results reported here need to be confirmed by aerosol experiments before extrapolating to humans.

Conclusions

To our knowledge, our study is the first one to envisage the toxicity effect of e-cigarette refill liquid on spermatogenesis in an animal model *in vivo*. The present data demonstrate that E-liquid 0% or E-liquid can produce adverse effects on sperm parameters in male rats. E-cigarette refill liquid, by promoting oxidative stress and inflammation, caused reproductive toxicity in the epididymis tissues, leading to spermatogenic modifications by decreasing the number and the viability of spermatozoa and increasing flagellum morphological abnormalities. As some flavoring agents, for instance cinnamon flavour (4), seem to be involved in e-liquid cytotoxicity, we cannot exclude diacetyl participation in epididymis toxicity. Further investigations are needed to understand how e-liquid per se can impact the reproductive system.

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Conflict of Interest

The authors declare that they have no conflicts of interest.

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