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ABSTRACT

Tobacco smoking is classified as a human carcinogen. A wide variety of new products, in particular electronic cigarettes (e-cigs), have recently appeared on the market as an alternative to smoking. Although the *in vitro* toxicity of e-cigs is relatively well known, there is currently a lack of data on their long-term health effects. In this context, the aim of our study was to compare, on a mouse model and using a nose-only exposure system, the *in vivo* genotoxic and mutagenic potential of e-cig aerosols tested at two power settings (18W and 30W) and conventional cigarette (3R4F) smoke. The standard comet assay, micronucleus test and *Pig-a* gene mutation assay were performed after subacute (4 days), subchronic (3 months) and chronic (6 months) exposure. The generation of oxidative stress was also assessed by measuring the 8-hydroxy-2'-deoxyguanosine and by using the hOGG1-modified comet assay. Our results show that only the high-power e-cig and the 3R4F cigarette induced oxidative DNA damage in the lung and the liver of exposed mice. In return, no significant increase in chromosomal aberrations or gene mutations were noted whatever the type of product. This study demonstrates that e-cigs, at high-power setting, should be considered, contrary to popular belief, as hazardous products in terms of genotoxicity in mouse model.

KEYWORDS

E-cigs

In vivo comet assay

In vivo micronucleus test

In vivo Pig-a gene mutation assay

Oxidative DNA damage

ABBREVIATIONS

e-cig: electronic cigarette; Mb18W: Modbox e-cig model set at 18 W; Mb30W: Modbox e-cig model set at 30 W; 8-OHdG: 8-hydroxy-2'-deoxyguanosine; hOGG1: human 8-oxoguanine glycosylase; *Pig-a*: phosphatidylinositol glycan, class A (gene); TI: tail intensity; MNPCE: micronucleated polychromatic erythrocytes; PCE: polychromatic erythrocytes; NCE: normochromatic erythrocytes; RET: reticulocytes; RBC: red blood cells; PAHs: polycyclic aromatic hydrocarbons; ALI: air-liquid interface.

1. INTRODUCTION

Tobacco use is a major public health concern killing more than 8 million people every year worldwide, yet is the leading cause of preventable death worldwide (World Health Organization, 2020). Cigarette smoke is composed of a mixture of toxicants and carcinogens such as polycyclic aromatic hydrocarbons (PAHs), N-nitrosamines, aromatic amines, aldehydes (*e.g.* formaldehyde and acetaldehyde), phenols, volatile hydrocarbons, and metals (International Agency for Research on Cancer, 2012, 2004). Tobacco smoking is classified as a human carcinogen (group 1) by the International Agency for Research on Cancer (IARC) for the development of mainly lung cancer and several other cancers (larynx, pharynx, oesophagus, stomach, colon, liver, pancreas, bladder, cervix, ...) (International Agency for Research on Cancer, 2012, 2004). It is also well known that smoking is a major risk factor for many other adverse effects on human health, including respiratory, cardiovascular, nervous, immune, liver, urinary, gastrointestinal and reproductive systems (Altamirano and Bataller, 2010; Dechanet et al., 2011; Erhardt, 2009; Gotts et al., 2019; Lakier, 1992; Li et al., 2014; Orth, 2000; Soares and Melo, 2008; Sopori, 2002; Sopori and Kozak, 1998). In recent years, a wide variety of new products, in particular electronic cigarettes (e-cigs), have emerged on the market as an alternative to smoking tobacco products. E-cigs are battery-powered devices that allow the nebulization of e-liquids composed of propylene glycol and/or glycerol and flavoring agents, with or without nicotine. E-cigs are generally perceived as less harmful than traditional cigarettes, particularly because they do not contain tobacco, do not require combustion during use and deliver fewer toxicants (Cao et al., 2021; Dusautoir et al., 2021). However, due to the thermal degradation of the e-liquid constituents, other substances in e-cig aerosols have been identified as toxic compounds or potential carcinogens such as aldehydes (*e.g.* formaldehyde, acetaldehyde, methylglyoxal, acrolein), phenolic compounds (*e.g.* phenol, quinol, catechol, ortho-, meta- and para-cresol), volatile organic compounds (*e.g.* xylene, toluene, acetonitrile) and heavy metals (*e.g.* nickel and copper) (Beauval et al., 2019, 2017, 2016; Cao et al., 2021; Erythropel et al., 2019; Gillman et al., 2016; Merecz-Sadowska et al., 2020; Polosa et al., 2019). Moreover, due to the lack of in-depth toxicity assessment, especially long term or repeated-dose toxicity studies, safety of e-cigs cannot be guaranteed.

Recent reviews (Cao et al., 2021; Merecz-Sadowska et al., 2020; Polosa et al., 2019; Wang et al., 2019) provide an overview of the *in vitro* and *in vivo* toxicity of e-cigs. To date, the published studies mainly focus on *in vitro* toxic effects of e-cigs emissions. Data show that exposure to e-cig aerosols

triggers cytotoxic effects such as cell death, DNA damage, and reactive oxygen species (ROS) and proinflammatory agents' production. It was also demonstrated that e-cig aerosol is much less cytotoxic than traditional cigarette smoke (Anthérieu et al., 2017; Cervellati et al., 2014; Dusautoir et al., 2021; Neilson et al., 2015; Tellez et al., 2021). *In vitro*, e-cigs had the potential to increase oxidative stress and inflammatory response in a similar level to that of cigarette smoke, but after more intensive exposures (Dusautoir et al., 2021). In contrast, there are relatively few *in vivo* experimental approaches. Most of them were performed or sponsored by the tobacco industry and have been carried out using short-term exposures (from a few hours to a few weeks), with individual e-cig components (*e.g.* popylene glycol and/or vegetable glycerine) but not whole aerosols, or with old generation or low-power e-cig devices. Furthermore, the experimental protocols of exposure often used did not correspond to normal conditions of use (*e.g.* number of puffs/min), or used whole body exposure systems that are not representative of real exposure since animals are exposed by other routes than the respiratory route (*i.e.* cutaneous and digestive by deposition of e-cig aerosols on the coat). Another limitation of most of these studies is that they did not compare the results obtained with e-cig aerosol with those obtained with traditional cigarette smoke. Results showed that e-cig aerosols are likely to induce oxidative stress, mitochondrial dysfunction, pulmonary inflammation, DNA damage and even impairment of respiratory function (Canistro et al., 2017; Garcia-Arcos et al., 2016; Glynos et al., 2018; Hwang et al., 2016; Laube et al., 2017; Lerner et al., 2016; Lim and Kim, 2014; McGrath-Morrow et al., 2015; Salturk et al., 2015; Scott et al., 2018; Werley et al., 2016).

Regarding the assessment of the genotoxicity of tobacco products, many studies have been conducted to specifically investigate the *in vitro* genotoxic/mutagenic potential of cigarette smoke and e-cig aerosols. Unfortunately, contradictory results have often been obtained depending on the products tested (*e.g.* particulate phase, gas phase, smoke condensate or extract, e-liquid itself, or whole smoke aerosol), the cell line used (*e.g.* lung epithelial cells, oral epithelial cells, oropharynx cells, or 'regulatory' cells), or the mode of cell exposure [(*e.g.* submerged cell cultures or air-liquid interface (ALI) conditions)]. For these reasons, some of these cell treatment/exposure methods are not representative of an actual human exposure to cigarette smoke or e-cig aerosols. In the Ames test, negative responses were observed with e-liquid and e-cig aerosols (with nicotine and a range of flavorings) whereas positive results were obtained concurrently with 3R4F smoke (Wieczorek et al., 2020). Similar negative results were

obtained with e-liquids and pad-collected aerosols of e-cigs, and positive results with pad-collected smoke condensates of tobacco cigarettes (3R4F, 1R5F, Malboro gold) (Misra et al., 2014). Rudd *et al.* also demonstrated that e-cig emission is not mutagenic under their tested conditions, unlike 3R4F cigarette smoke (Rudd et al., 2020). Thorne *et al.* have carried out a study on *Salmonella typhimurium* strains TA98 and TA100 exposed at the air-agar interface to e-cig aerosols and showed no mutagenic activity in contrast to 3R4F cigarette smoke (Thorne et al., 2016). The same authors also performed a mouse lymphoma assay at the tk locus and *in vitro* micronucleus tests (on CHO, V79 and TK6 cells) with an e-liquid, the e-cig aerosol matter captured from the same e-liquid, and the total particulate matter from a 3R4F cigarette. No mutagenic or genotoxic effect was observed for the e-liquid and its aerosol, in contrast to 3R4F smoke (Thorne et al., 2019a, 2019b). All *in vitro* micronucleus tests reported in the literature were negative (Misra et al., 2014; Rudd et al., 2020; Tellez et al., 2021; Thorne et al., 2019a; Wieczorek et al., 2020), either with e-liquids, aerosols or condensates, except the one reported very recently by Tellez *et al.* (2021) on e-cig aerosols (containing diverse flavoring product, with and without nicotine) in oral epithelial cells. In contrast, in all these studies, traditional cigarette smoke (or total particulate matter or condensate) induced chromosomal aberrations. Very recently, Tellez *et al.* demonstrated that 10 different e-cig aerosols did not induce DNA damage, as measured by the *in vitro* comet assay, in oral epithelial cells, unlike the 3R4F cigarette (Tellez et al., 2021). This result was not confirmed by several previously published data. Khalil *et al*., also using the *in vitro* comet assay, showed that e-cig aerosols cause DNA damage in A549 lung cells exposed at the ALI (Khalil et al., 2021). Ganapathy *et al.* also reported that e-cig aerosol extracts can induce significant increases in DNA damage (using the primer anchored DNA damage detection assay), including 8-OHdG, on human oral and lung epithelial cells (Ganapathy et al., 2017). Yu *et al.* observed increases in DNA strand breaks (as measured by the *in vitro* comet assay and the γH2AX immunostaining) after short- and long-term exposure (48 hours to 8 weeks) to e-cig aerosol extracts, on several normal and cancerous cell lines (Yu et al., 2016). Finally, two studies performed on A549 and/or BEAS-2B pulmonary cells exposed at the ALI to whole smoke from reference cigarettes (M4A and/or 3R4F) reported the induction of DNA damage using the γH2AX assay or the *in vitro* comet assay (Garcia-Canton et al., 2014; Weber et al., 2013).

In vivo genotoxicity studies are much less numerous and less recent. Almost all of them have been carried out on cigarette smoke and results show induction of micronuclei in bone marrow, peripheral blood and lung of exposed rodents, as well as DNA damage in lung, stomach and liver cells (Balansky,

1999; Balansky et al., 2000; D'Agostini et al., 2001; Dalrymple et al., 2016, 2015; Nakamura et al., 2015; Tsuda, 2000; Ueno et al., 2011). Some negative results have been also reported in the micronucleus and the *Pig-a* mutation tests. In contrast, there is very little *in vivo* genotoxicity data on e-cigs with only two published studies. Canistro *et al.* found that e-cig aerosol increased DNA damage and micronuclei formation in peripheral blood of rats exposed for 4 weeks, and the collected urine of animals induced reverse mutations in the Ames test (Canistro et al., 2017). Using the ^{32}P-postlabeling method, Lee *et al.* showed that e-cig emission induced DNA adducts in lung, bladder and heart tissues of exposed mice (Lee et al., 2018).

Because of a daily and prolonged consumption of e-cig by many users, it is now essential to produce data on the mechanisms underlying the potential genotoxicity of e-cigs after long-term exposure. In this context, the aim of our study was to investigate the *in vivo* genotoxic and mutagenic effects of e-cig aerosols compared to traditional cigarette smoke. After nose-only exposure of BALB/c mice, the *in vivo* genotoxic and mutagenic potential of smoke from conventional cigarette (3R4F) and emissions from a "Modbox" e-cig model with 0.5 Ohms coil and set at 18W (Mb18W) or 30W (Mb30W) power were assessed using (i) the *in vivo* comet assay in lung (primary target organ) and liver (systemic and most active metabolizer organ), (ii) the *in vivo* micronucleus test in bone marrow and (iii) the *in vivo Pig-a* gene mutation assay in peripheral blood (to identify possible systemic effects). The standard comet assay was performed within the framework of subacute (4 days), subchronic (3 months) and chronic (6 months) exposures. The micronucleus test and the *Pig-a* gene mutation assay, as markers of effects, were only carried out for the 3- and 6-month exposures. For ethical and scientific reasons, these three tests were applied to the same animals. In order to specifically determine oxidation-dependent DNA damage, we also measured the pulmonary 8-OHdG content after subacute, subchronic and chronic exposures, and the results were confirmed by performing a modified comet assay using the human 8-oxoguanine glycosylase (hOGG1) after the 6-month treatment.

2. MATERIALS AND METHODS

2.1. E-cigarettes, e-liquid and conventional cigarette

Today there is a wide variety of e-cigs and e-liquids. As explained in our previous studies (Beauval et al., 2019; Dusautoir et al., 2021), we chose the third generation “ModBox” model, used with the “Air Tank” clearomiser equipped with a 0.5 Ω kanthal coil and with a partially closed air flow. For our experiments, we chose two power settings for the Modbox model: a “low” power of 18W and a “high” power of 30W. Both devices are from NHOSS® (Innova, Bondues, France). For the e-liquid, we chose the best-selling NHOSS® brand containing 65% propylene glycol, 35% glycerine, 16 mg/mL nicotine and the most common flavour, “blond tobacco”, representative of a standard e-liquid in accordance with the French national organisation for standardisation (AFNOR) recommendations (AFNOR, Association Française de Normalisation, 2015). Conventional 3R4F cigarettes were obtained from the University of Kentucky (Lexington, KY, USA).

2.2. Animal model

Experiments were conducted on male BALB/c mice (Janvier Labs, Le Genest-Saint-Isle, France), 9 weeks old, 5 animals/group. This mouse strain is described as sufficiently sensitive to the chemical induction of lung cancers (Meuwissen, 2005). Animal procedures were in agreement with European directive 2010/63/EU for the protection of animals used for scientific purposes and obtained the Ethical Committee on Animal Experimentation (CEEA 75) approval.

2.3. Aerosol generation and mice exposure protocols

To avoid chemical cross-contamination, two different pieces of equipment (exposure towers and pipes) were used for e-cig and 3R4F exposures. Aerosols from e-cigs and 3R4F cigarette were generated with an InExpose e-cigarette extension system on which we adapted the Modbox and a cigarette smoking robot (SCIREQ®, Emka technologies, Montreal, Quebec, Canada), respectively. Mice were exposed to aerosols by a nose-only tower (InExpose system, SCIREQ®, Emka technologies). In order to perform a comparative study of the *in vivo* genotoxicity of the e-cig aerosols and tobacco cigarette smoke, all products were tested with Health Canada Intense puff profile (55 mL puff volume, 2 s puff duration, 30 s puff period).

Based on data from the literature and our preliminary study after a 4-day subacute exposure (data not shown), three exposure protocols were applied in this study (Table 1). First, a subacute exposure for 4 days (4 treatments at 24-hour intervals for 30, 60 or 90 min/day for both e-cigs, and for 60 min/day for 3R4F) was performed as a preliminary toxicity assessment. Then, a 3-month subchronic and a 6-month chronic exposure were realized (60 min/day, 5 days/week for e-cigs and 3R4F).

For each exposure schedule, one group was sham-exposed to fresh conditioned air (negative control). Control groups with genotoxic reference compounds were also used for *in vivo* genotoxicity studies (see part 2.5).

Animal body weights were recorded on Monday of each weak while clinical signs were monitored daily (data not shown).

2.4. Chemical characterization of aerosols

Chemical composition of aerosols from electronic and conventional cigarettes was assessed and described in our previous study (Dusautoir et al., 2021). Chemical characterization analyses focused on the quantification of nicotine and the identification and quantification of carbonyl compounds and PAHs (see part 4).

2.5. *In vivo* genotoxicity assessment

The genotoxic/mutagenic potential of conventional and electronic cigarettes emissions was assessed after subacute (4 consecutive days), subchronic (3 months) and chronic (6 months) exposures by using a battery of three *in vivo* tests, namely the comet assay, the micronucleus assay and the *Pig-a* gene mutation assay. These studies were carried out using an approach very similar to that of Good Laboratory Practice (GLP). Tests, endpoints, target organs and treatment schedules are summarized in Table 1.

2.5.1. *In vivo* comet assay

The *in vivo* comet assay was performed in isolated lung and liver cells under alkaline conditions (pH>13) according to previously described protocol (Platel et al., 2020; Singh et al., 1988; Tice et al., 2000; Witte et al., 2007) and in compliance with the OECD test guideline No. 489 (OECD, 2016a). At the end of each exposure period, a positive control group was treated orally with methyl methanesulfonate

(MMS) [100 mg/kg body weight (b.w)/day for 2 consecutive days in sterile water]. For all groups (*i.e.* treated and controls), tissues were collected once at 2-6 h after the last treatment. For the 6-month exposure time, slight modifications were added (use of hOGG1) to specifically detect oxidative DNA damage, based on Collins' and Smith's procedures (Collins et al., 1993; Smith et al., 2006). 750 randomly selected cells per group (*i.e.* 50 cells per slide, 3 slides per animal, 5 animals per group) were analysed for DNA fragmentation scoring using the Comet Assay IV Image Analysis System, version 4.11 (Perceptive Instruments Ltd, Suffolk, United Kingdom). DNA damage was expressed as percentage of DNA in the tail (% tail intensity) (Burlinson *et al.*, 2007; Lovell and Omori, 2008).

2.5.2. *In vivo* micronucleus test

The *in vivo* micronucleus test was performed in the bone marrow of treated mice in compliance with the OECD test guideline No. 474 (OECD, 2016b). A positive control group was treated orally with MMS [100 mg/kg b.w/day (x2 days) in sterile water] (see part 2.5.1). The protocol has been previously described (Platel et al., 2020). Two slides per animal were prepared. For the determination of genotoxicity, slides were blindly scored by microscopy for the number of polychromatic erythrocytes (PCE) (2000 PCE per slide, *i.e.* 4000 PCE per animal) having one or more Howell-Jolly bodies (micronucleated polychromatic erythrocytes, MNPCE). For the determination of cytotoxicity, the polychromatic/normochromatic erythrocyte ratio (PCE/NCE) was determined from the microscopic examination of at least 500 erythrocytes per slide (*i.e.* 1000 erythrocytes per animal).

2.5.3. *In vivo Pig-a* gene mutation assay

The quantification of *in vivo Pig-a* (phosphatidylinositol glycan, class A) gene mutation (Bryce et al., 2008; Dobrovolsky et al., 2010; Kimoto et al., 2011) was performed with the MutaFlow^{PLUS} Kit Mouse Blood (Litron, Rochester, New York) as previously described (Platel et al., 2020). According to the kinetics for mutant phenotype cells appearance in circulation and ease of scoring, one month before the end of each exposure period, a positive control group was treated orally with ethyl-nitrosourea (ENU) [40 mg/ kg b.w/day for 3 consecutive days in sterile water]. Blood samples were collected after 3 and 6 months of exposure. The incidence of *Pig-a* mutation per animal was expressed as the number of CD24-negative red blood cells (RBC) per one million RBC, and as the number of CD24-negative reticulocytes (RET) per

297 one million RET, using a FACSCanto II flow cytometer (BD Biosciences) running FACSDiva™ v7.0
298 software. The percentage of RET was also established for cytotoxicity assessment.
299
300 **2.6. 8-hydroxy-2'-deoxyguanosine (8-OHdG) assay**
301 8-OHdG level was measured in the genomic DNA of mouse lung tissues following 4 days, 3 months or 6
302 months of exposure to either electronic or conventional cigarette aerosols. Genomic DNA was extracted
303 using the QIAamp DNA mini kit (Qiagen, Courtaboeuf, France) following manufacturer's
304 recommendations. Extracted DNA was pre-treated with P1 nuclease using the reagents from Wako 8-
305 OHdG Assay Preparation (Wako, Tokyo, Japan). This step permits to digest the DNA down to the single
306 nucleotide level. 8-OHdG level were then determined using a competitive enzyme-linked immunosorbent
307 assay (ELISA): Oxiselect™ Oxidative DNA Damage Kit (Cell Biolabs, San Diego, CA), according to the
308 manufacturer's recommendations. Results were expressed as fold-change (± SD) relative to the 8-OHdG
309 level in control mice arbitrarily set at a value of 1.
310
311 **2.7. Statistical Analysis**
312 All statistical analyses were performed with GraphPad InStat® Software (version 3.10). For each
313 test, differences between groups (*i.e.* between each concentration *vs.* the respective negative control) with
314 $p<0.05$ were considered statistically significant.
315 The Mann-Whitney U-test was used for the comet assay, the micronucleus test (for the frequency of
316 MNPCE) and the 8-OHdG content. The Student's t test was used for the statistical comparison for the
317 PCE/NCE ratio (micronucleus test). The Dunnett's t-test (pair-wise comparison) was performed for the
318 *Pig-a* gene mutation assay.
319

3. RESULTS

Results of the *in vivo* tests are summarized in Table 2. For each test, concurrent negative controls (animals sham-exposed to fresh conditioned air) were within the range of current observed values and concurrent positive controls induced responses that are comparable to the historical positive control data (data not shown) and produced a statistically significant increase compared with the negative control. The validity criteria for the tests were considered as fulfilled.

3.1. Subacute exposure (4-day treatment)

The genotoxic potential of electronic and conventional cigarettes was investigated in the *in vivo* comet assay on isolated lung and liver cells of mice after a subacute exposure (4 treatments at 24-hour intervals for 90 min/day for both e-cigs, and for 60 min for 3R4F). Results of the means of medians of percentage of tail intensity (TI) are given in Figure 1. Under tested conditions, no increase in DNA strand breaks was observed in the two selected organs, for both conventional and electronic cigarettes.

Regarding the levels of 8-OHdG in mouse lung tissues, exposure to cigarette smoke for 60 min, 4 days in a row, induced a significant increase relative to air-exposed mice (1.6 fold-change) (Figure 4A). For e-cigs, subacute exposure to Mb18W aerosol induced no change in 8-OHdG levels regardless of the duration of exposure (*i.e.* 30, 60 or 90 min), whereas exposures to Mb30W emissions for 60 min and 90 min induced a statistically significant increase compared to the control (1.5 and 1.6 fold-changes) (Figure 4A).

3.2. Subchronic exposure (3-month treatment)

Results of genotoxicity/mutagenicity assessment after the 3 months subchronic exposure of mice (60 min/day, 5 days/week) are presented in Figure 2.

For both e-cigs and the conventional cigarette, no statistically significant increase in the level of DNA damage was observed, in either the liver (Figure 2A) or the lung (Figure 2B). The highest TI was obtained with Mb30W in the liver (2.3 % *vs*. 1.69 % for the negative control).

Regarding the frequency of MNPCE, no significant increase was found in animals exposed to Mb18W (0.75 ‰), Mb30W (1.13 ‰) or 3R4F (0.60 ‰) emissions when compared to the control group (0.55 ‰) (Figure 2C). The ratio PCE/NCE was not significantly affected by exposure to e-cig aerosols and

3R4F cigarette smoke, indicating the absence of cytotoxic effects (a very slight decrease but non-statistically significant was observed with 3R4F).

The frequencies of mutants RET (highest value: 2.92 x 10^{-6} for 3R4F) and mutants RBC (highest value: 3.42 x 10^{-6} for 3R4F) did not show statistically significant increase in the animals exposed to Mb18W, Mb30W or 3R4F emissions when compared to the control group exposed to air (RET = 1.44 x 10^{-6} and RBC = 2.08 x 10^{-6}) (Figure 2D). The percentage of RET is the ratio of newly formed RNA-positive erythrocyte relative to all erythrocytes, and is used as a measure of bone marrow cytotoxicity. E-cig and conventional cigarette exposed mice did not exhibit significant changes in % RET after a 3-month exposure, thus confirming the absence of toxicity.

After 3 months of exposure to Mb30W aerosol and 3R4F smoke, 8-OHdG quantity assessment in DNA of mouse lung tissues showed a statistically significant increase compared to control (1.8 and 2.0 fold-changes, respectively) while an exposure to Mb18W emissions induced no change (Figure 4B).

3.3. Chronic exposure (6-month treatment)

Results obtained after the 6-month chronic exposure (60 min/day, 5 days/week) are presented in Figure 3.

In the standard comet assay, no increase in DNA strand breaks was observed for both conventional and electronic cigarettes. On the contrary, with the hOGG1-modified comet assay, statistically significant increases ($p<0.05$) in TI were observed for Mb30W and 3R4F in the liver (15.15 % and 11.46 %, respectively, *vs.* 1.52 % for the negative control) (Figure 3A) and in the lung (34.96 % and 30.59 %, respectively, *vs.* 11.57 % for the negative control) (Figure 3B), indicating oxidative DNA damage induction.

Under tested conditions, no induction of MN formation was observed in mice exposed to e-cigs 18W, 30W or 3R4F cigarette aerosols (< 0.8 ‰). No decrease of the ratio PCE/NCE was observed (Figure 3C).

No statistically significant increase in mutant frequencies of RBC (highest value: 0.37 x 10^{-6} cells for 3R4F) and RET (highest value: 0.47 x 10^{-6} cells for 3R4F) was observed whatever the types of cigarette compared to the control group (RET mutant frequency = 1.33 x 10^{-6} and RBC mutant frequency = 0.65 x 10^{-6}) (Figure 3D). The % RET was not significantly affected indicating the absence of toxic effects in the bone marrow at this exposure level.

379 Consistent with subacute and subchronic exposures, a 6-month exposure to Mb18W aerosol
380 induced no change in the level of 8-OHdG in the lung tissue DNA of mice compared to air-exposed mice,
381 whereas Mb30W aerosol and 3R4F smoke induced a statistically significant increase (1.2- and 1.4-fold,
382 respectively) (Figure 4C).
383

4. DISCUSSION

Electronic nicotine delivery systems are considered by public opinion to be less harmful than traditional cigarettes, and are currently used as a smoking cessation aid. Paradoxically, there is a lack of long-term *in vivo* studies on their health effect, thus their safety cannot be claimed. To fill this gap, we carried out a comprehensive assessment of the *in vivo* genotoxicity and mutagenicity of an e-cig model set to two different power levels (18W and 30W) and of conventional cigarette. The conditions of animal exposure, in terms of route (pulmonary), mode (nose-only), time (short and long-term treatment) and puff profile (Health Canada Intense profile), were designed to be as close as possible to human vaping conditions.

Under our experimental conditions, whatever the duration of animal exposure, 3R4F cigarette and e-cigs at both powers did not induce an increase in DNA strand breaks in lung and liver cells, as measured by the standard comet assay. This result may seem in contradiction with the study carried out by Canistro *et al.* in which e-cig aerosol produced DNA damage in leukocytes of whole-body exposed rats (Canistro et al., 2017). However, as the authors themselves stated, their data should be analysed with caution as the exposure conditions used [animals were submitted to 11 cycles (puff: 6s on, 5s off, 6s on)/day, 5 days/week, for 4 weeks] did not reflect actual human exposure to e-cig aerosols. Their aim was to characterize a hazard and perhaps the use of too high doses may explain the induction of non-specific DNA damage. Other published data have also shown positive results in the *in vivo* comet assay on stomach, liver and/or lung with cigarette smoke (Tsuda, 2000; Ueno et al., 2011).

On the other hand, our results showed that only Mb30W and 3R4F aerosols induced a statistically significant increase in 8-OHdG formation in the lung of exposed mice after 4 days, 3 months and 6 months of exposure. At the end of our study (*i.e.* for the 6-month exposure) we decided to confirm this result by using a modified protocol for the comet assay. Indeed, we used the repair endonuclease hOGG1 to better characterize the mechanism of genotoxicity of e-cig emissions and conventional cigarette smoke. The hOGG1-modified comet assay is a useful tool to increase both the sensitivity and the specificity of the test and thus provide first elements of the oxidizing mode of action of test compounds (Platel et al., 2011). The corresponding results were consistent with the 8-OHdG measurement since only Mb30W and 3R4F aerosols induced significant oxidative DNA damage in the lung and the liver of exposed mice. Our findings are also in line with our previous study (Dusautoir *et al.*, 2021) and with reviews reporting that exposure to

e-cig aerosols is related to oxidative stress (Cao et al., 2021; Merecz-Sadowska et al., 2020; Polosa et al., 2019; Wang et al., 2019). Interestingly, Dalrymple *et al.* also showed, after 5 days of nose-only exposure of rats to 3R4F cigarette smoke, an increase in oxidative DNA damage in alveolar type II lung cells exclusively by using the FPG-modified comet assay (*i.e.* no DNA damage was observed with the classical protocol without FPG) (Dalrymple et al., 2015). The authors also found oxidative DNA damage after 3 and 6 weeks of exposure (Dalrymple et al., 2016).

Very recently, we have carried out a comparison of the chemical composition of aerosols from Mb18W, Mb30W and 3R4F (Dusautoir *et al.*, 2021). We showed that increasing the power of the e-cig can induce an increase in the amount of toxic compounds in the aerosol (by puff, Mb18W emitted 6.9% and 51.4% less total PAHs and carbonyl compounds, respectively, than Mb30W). It has been previously demonstrated that higher power leads to higher carbonyls compounds production due to higher coil temperature (up to 300°C) and thus the thermal degradation of e-liquid and that, secondarily, the increased level of carbonyl compounds results in the formation of ROS (Dusautoir et al., 2021; Geiss et al., 2016; Haddad et al., 2019; Kosmider et al., 2014; Zhao et al., 2018). Our results are thus consistent with these explanations since in our study oxidative DNA damage was observed only with Mb30W.

Noteworthy, we observed an almost similar response between the 3R4F cigarette and the Mb30W e-cig. It is difficult, if not impossible, to define precisely which toxic substance(s) is (are) responsible for the genotoxic effect observed in each case. The use of predictive toxicity methods (*i.e. in silico* models) would be an interesting tool for this purpose as an alternative approach to experimental testing. In the study performed by Barhdadi *et al.*, a genotoxic alert was identified by (Q)SAR models for 60 flavoring substances identified among the 129 e-liquids tested (Barhdadi et al., 2021). Based on information collected from EU databases 5 flavoring substances of genotoxic concern were identified (estragole, safrole, 2,5-dimethyl-4-hydroxyl-3(2H)-furanone, furylmethylketon and trans-hexenal) and 4 substances (2,3-butanedione, 2,3-pentanedione, isoledene and β-phellandrene) gave positive result in at least one *in vitro* test (Ames and/or *in vitro* micronucleus test). Similarly, Kang *et al.* used (Q)SAR models to predict DNA adducts formation by flavor chemicals found in e-liquid and e-cig aerosols (Kang and Valerio, 2020). Two chemical classes were identified, alkenylbenzenes (including estragole and eugenol) and aldehydes (including acrolein, glyoxal and methylglyoxal), well known to be produced in cigarette smoke and e-cig aerosol (Beauval et al., 2019; Bekki et al., 2014; Dusautoir et al., 2021; Hutzler et al., 2014; Khlystov and Samburova, 2016; Peace et al., 2018).

Lee *et al.*, as a step towards understanding the carcinogenicity of e-cig aerosols, demonstrated that nicotine (noncarcinogenic in animals) can be nitrosated, metabolized, and further transformed into methyldiazohydroxide (MDOH) and aldehydes in lung, bladder, and heart tissues of mice (Lee et al., 2018). They found that aldehydes and MDOH induced DNA adducts and also decreased DNA repair. Interestingly, we previously showed that the level of nicotine delivered in the aerosols is much lower for Mb18W (60 µg/puff) than for Mb30W (137 µg/puff) and 3R4F (95 µg/puff) (Dusautoir *et al.*, 2021). Therefore, it can be assumed that the level of DNA adducts to be formed could be less for Mb18W which is consistent with our results.

In addition, our results revealed that both traditional cigarette smoke and e-cig aerosol induced no biologically or statistically significant increases in chromosomal aberrations and gene mutations, whatever the duration of exposure. Therefore, they are considered having no mutagenic activity under our experimental conditions. These results, although at first sight surprising, are fully in line with those of Dalrymple *et al.* (2016). In their study, rats were nose-only exposed to 3R4F cigarette (1h or 2h/day, 5 days/week) for 3 and 6 weeks. Blood was collected only at the 6-week timepoint and results showed that *Pig-a* gene mutations and micronucleus frequencies were not significantly increased (as mentioned above, positive results were obtained in the modified comet assay). Others have also obtained negative results in the *in vivo* micronucleus test on bone marrow or peripheral blood following nose-only cigarette exposure (Schramke et al., 2014; Van Miert et al., 2008). For e-cig, no data was found in the literature regarding the assessment of its *in vivo* mutagenicity, with the exception of the study by Canistro *et al.* (2017) that showed micronuclei formation in reticulocytes of rats whole-body exposed to e-cig aerosol. However, as explained above, their data should be compared with ours with caution because the exposure conditions they used were not intended to reflect actual human exposure to e-cig emission but rather to characterize a hazard. Indeed, in our study, the absence of mutagenic effect of reference cigarette smoke and e-cig aerosols, while it is well known that they are composed of carcinogenic substances, suggest that the experimental conditions we implemented, although realistic, may not be high enough to reach a level of exposure in bone marrow and blood to induce a positive response in the micronucleus and *Pig-a* tests, respectively. Furthermore, as already mentioned by Dalrymple *et al.* (2016) it is possible that cigarette and e-cig do not induce mutagenic effect in organs other than the respiratory system (*i.e.* the first tissue of contact and target organ of tobacco products). Another important point that

may explain the negative results is the sensitivity of the tests. Although the *in vivo* micronucleus test in bone marrow or peripheral blood is traditionally the most used *in vivo* test in the first instance, it is known to have a poor sensitivity of about 40-50% (Benigni et al., 2010; Kirkland and Speit, 2008; Morita et al., 2016). As there is no single 'ideal' test for detecting clastogenic, aneugenic and mutagenic genetic events, it is common to use a combination of several tests (different genotoxic endpoints), as we did in our study, to increase sensitivity without reducing specificity. The *Pig-a* test is known for its remarkable sensitivity to mutagenic agents (Gollapudi et al., 2015) and its relatively sensitivity to clastogens (Bhalli et al., 2013). Ideally, these tests should have been carried out on the lung and liver (*i.e.* on the target organs), but for methodological reasons this is not feasible. Despite an inter-laboratory study showed that the combination of the comet, micronucleus and *Pig-a* assays, using the same animals, may be a robust strategy for evaluating *in vivo* genotoxicity (Chung et al., 2018), it would have been relevant to perform an *in vivo* gene mutation test on the target organs (*i.e.* liver and lung) using transgenic animals. Indeed, transgenic rodent gene mutation tests have the ability to detect and quantify mutations in virtually all somatic tissues (Gingerich et al., 2014; Lambert et al., 2005; OECD, 2020). However, these tests are complex, currently expensive and not widely available.

5. CONCLUSION

The e-cig was initially developed as an alternative to conventional cigarette although there is insufficient data to assess its long-term safety for human health. In this context, our study was implemented with the aim of comparing the *in vivo* genotoxic and mutagenic potential of two low- and high-power e-cigs and the traditional cigarette, after subacute (4 days), subchronic (3 months) and chronic (6 months) exposure. In order to be as close as possible to human exposure conditions, animals were exposed to realistic doses of e-cig and cigarette emissions (*i.e.* Health Canada Intense puff profile) *via* the pulmonary route (nose-only). Under these experimental conditions, the main result of our study is that both 3R4F and Mb30W induce oxidative DNA damage in lung and liver, demonstrating that high-power e-cig should be considered as "hazardous material" as traditional cigarette, whereas e-cig at low power setting seems to be devoid of *in vivo* genotoxic effect. These differences in results between Mb18W and Mb30W are probably attributable to lower concentrations of toxic substances (mainly carbonyls compounds) in low power e-cig aerosols, as previously described. Moreover, micronuclei and *Pig-a* gene mutation were not detected in reticulocytes. This suggests that our experimental conditions, although realistic, may not be sufficient to reach a level of exposure in bone marrow and blood to induce a positive response. This also raises the question of the sensitivity of these two tests in organs other than the target organ (here the lung). It is important to underline the originality of our work which is based on a complete study of the *in vivo* genotoxic/mutagenic potential of e-cig. Finally, our work could be completed by assessing gene mutations in the target organs (*i.e.* liver and lung) using the transgenic rodent mutation assay. It would also be interesting to study other non-genotoxic endpoints involved in the potential carcinogenesis of e-cig such as epigenetic alterations. All these data could lead to a better regulation of these new alternatives to conventional cigarettes.

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7. DECLARATION OF COMPETING INTEREST

The authors declare that they have no conflict of interest with tobacco or e-cig industries.

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TITLE AND LEGEND OF TABLES AND FIGURES

Figure 1. Results of the *in vivo* comet assay after subacute exposure of mice to e-cig and conventional cigarette aerosols.

Animals (n=5) were exposed to conventional cigarette (3R4F) smoke for 60 min/day or to e-cig (Mb18W and Mb30W) emissions for 90 min/day, for 4 consecutive days. The negative control group was exposed to air. MMS [(100 mg/kg b.w./day)x2] was used as positive control. The level of DNA fragmentation on liver **(A)** and lung **(B)** cells is expressed as the mean of medians of % of tail DNA intensity (±SD). ** $p<0.01$ (Mann-Whitney U-test).

Figure 2. Results of *in vivo* genotoxicity/mutagenicity assessment after subchronic exposure of mice to e-cig and conventional cigarette aerosols.

Animals (n=5) were exposed to conventional cigarette (3R4F) smoke or to e-cig (Mb18W and Mb30W) emissions for 60 min/day, 5 days/week, for 3 months. The negative control group was exposed to air. MMS [(100 mg/kg b.w./day)x2] and ENU [(40 mg/kg b.w./day)x3] were used as positive controls. **(A-B)** The level of DNA fragmentation is expressed as the mean of medians of % of tail DNA intensity (±SD). **(C)** The chromosomal aberrations frequency is expressed as the number of micronucleated polychromatic erythrocytes (MNPCE) per 1000 cells (±SD). The polychromatic erythrocytes (PCE) / normochromatic erythrocytes (NCE) ratio is used as a measure of bone marrow cytotoxicity. **(D)** The gene mutations frequency is expressed as the number of red blood cells (RBC) or reticulocytes (RET) per 10^6 cells (±SD). Toxicity in bone marrow was measured by % RET. * $p<0.05$; ** $p<0.01$ (Mann-Whitney U-test for the comet assay and the micronucleus test, Dunnett's t-test for the *Pig-a* test).

Figure 3. Results of *in vivo* genotoxicity/mutagenicity assessment after chronic exposure of mice to e-cig and conventional cigarette aerosols.

Animals (n=5) were exposed to conventional cigarette (3R4F) smoke or to e-cig (Mb18W and Mb30W) emissions for 60 min/day, 5 days/week, for 6 months. The negative control group was exposed to air. MMS [(100 mg/kg b.w./day)x2] and ENU [(40 mg/kg b.w./day)x3] were used as positive controls. **(A-B)**

The level of DNA fragmentation is expressed as the mean of medians of % of tail DNA intensity (±SD). **(C)** The chromosomal aberrations frequency is expressed as the number of micronucleated polychromatic erythrocytes (MNPCE) per 1000 cells (±SD). The polychromatic erythrocytes (PCE) / normochromatic erythrocytes (NCE) ratio is used as a measure of bone marrow cytotoxicity. **(D)** The gene mutations frequency is expressed as the number of red blood cells (RBC) or reticulocytes (RET) per 10^6 cells (±SD). Toxicity in bone marrow was measured by % RET. * $p<0.05$; ** $p<0.01$ (Mann-Whitney U-test for the comet assay and the micronucleus test, Dunnett's t-test for the *Pig-a* test).

Figure 4. Results of *in vivo* lung 8-OHdG assessment in mice after acute, subchronic and chronic exposure to e-cig and conventional cigarette aerosols.

Animals were exposed to conventional cigarette (3R4F) or to e-cig (Mb18W and Mb30W) emissions for 30, 60 or 90 min/day for 4 days for subacute exposures (n = 5) and for 60 min/day, 5 days/week for 3 or 6 months for subchronic and chronic exposures (n = 8), respectively. The control group was exposed to air. The level of 8-OHdG is expressed as fold-change relative to the level found in control mice (± SD) measured using a competitive ELISA assay following 4 days **(A)**, 3 months **(B)** or 6 months **(C)** of exposure. *$p<0.05$ (Mann-Whitney U-test).

Table 1. Summary of *in vivo* genotoxic/mutagenic tests performed.

For subacute exposure, mice received 4 treatments at 24-hour intervals for 90 min (and for 30 and 60 min for the 8-OHdG assay) for e-cigs, and for 60 min for conventional cigarette. For subchronic (3 months) and chronic (6 months) exposures, animals were exposed to e-cig or 3R4F cigarette emissions for 60 min, 5 times a week. X: test performed.

Table 2. Summary of *in vivo* genotoxicity/mutagenicity tests results.

For subacute exposure, mice were exposed 4 times at 24-hour intervals for 90 min (and for 30 and 60 min for the 8-OHdG assay) to Mb18W and Mb30W aerosols, and for 60 min to 3R4F smoke. For subchronic (3

months) and chronic (6 months) exposures, animals were exposed 60 min/day, 5 days/week, to Mb18W, Mb30W and 3R4F aerosols. n.a.: not assessed; -: negative result; +: positive result.

FIGURE 1

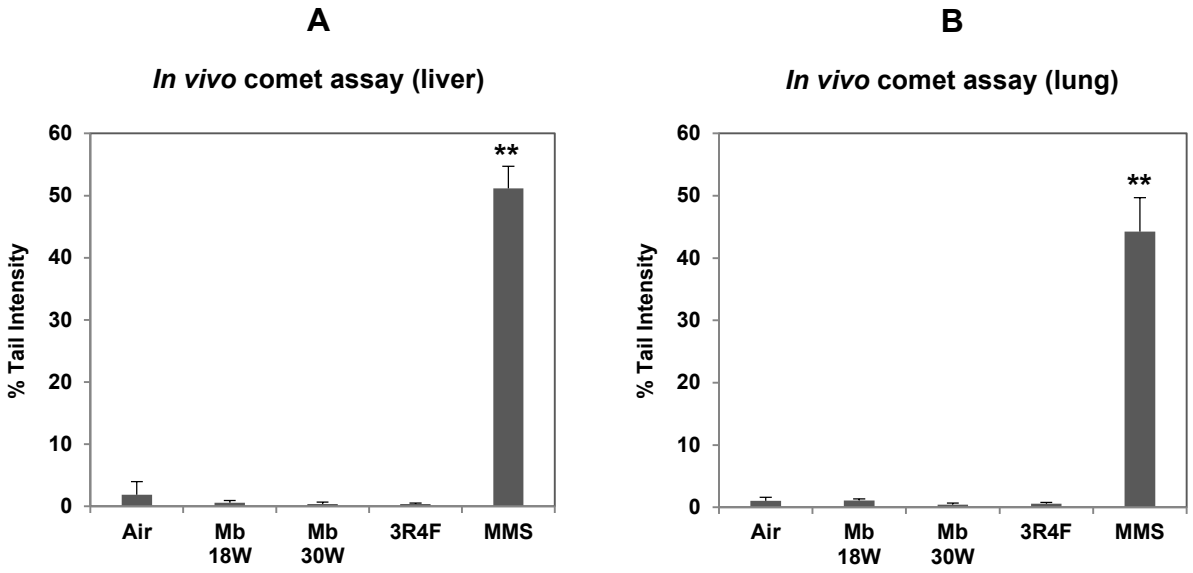

A
In vivo comet assay (liver)
% Tail Intensity
60
50
40
30
20
10
0
**
Air
Mb 18W
Mb 30W
3R4F
MMS
B
In vivo comet assay (lung)
% Tail Intensity
60
50
40
30
20
10
0
**
Air
Mb 18W
Mb 30W
3R4F
MMS

FIGURE 2

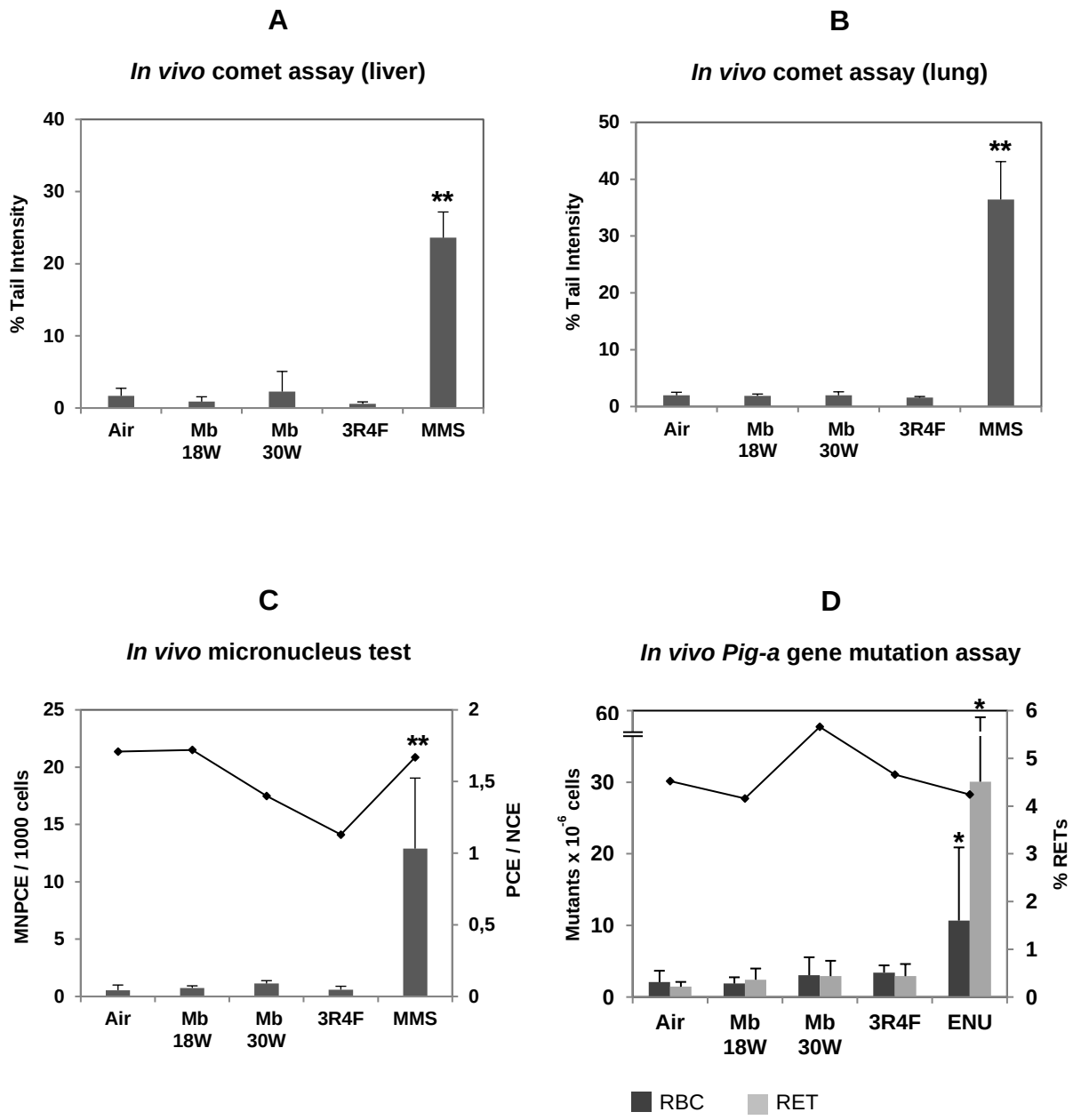
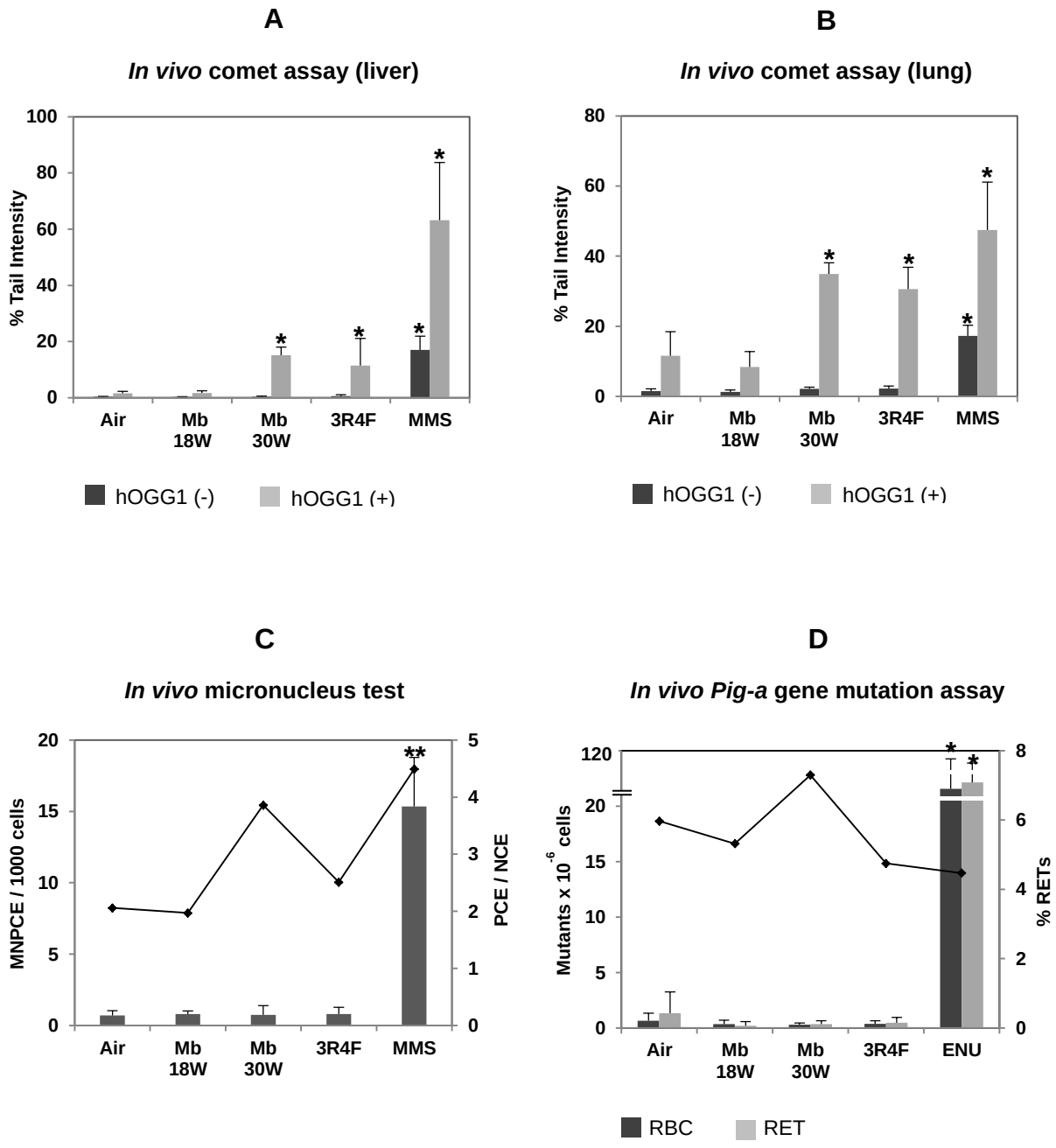
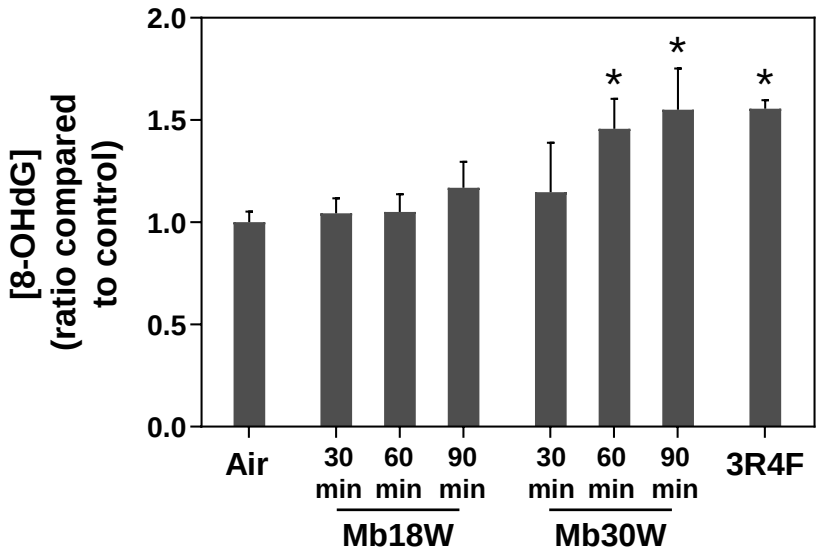

A
In vivo comet assay (liver)
% Tail Intensity
40
30
20
10
0
**
Air
Mb 18W
Mb 30W
3R4F
MMS
B
In vivo comet assay (lung)
% Tail Intensity
50
40
30
20
10
0
**
Air
Mb 18W
Mb 30W
3R4F
MMS
C
In vivo micronucleus test
MNPCE / 1000 cells
25
20
15
10
5
0
PCE / NCE
2
1,5
1
0,5
0
**
Air
Mb 18W
Mb 30W
3R4F
MMS
D
In vivo Pig-a gene mutation assay
Mutants x 10-6 cells
60
30
20
10
0
% RETs
6
5
4
3
2
1
0
*
*
Air
Mb 18W
Mb 30W
3R4F
ENU
RBC
RET

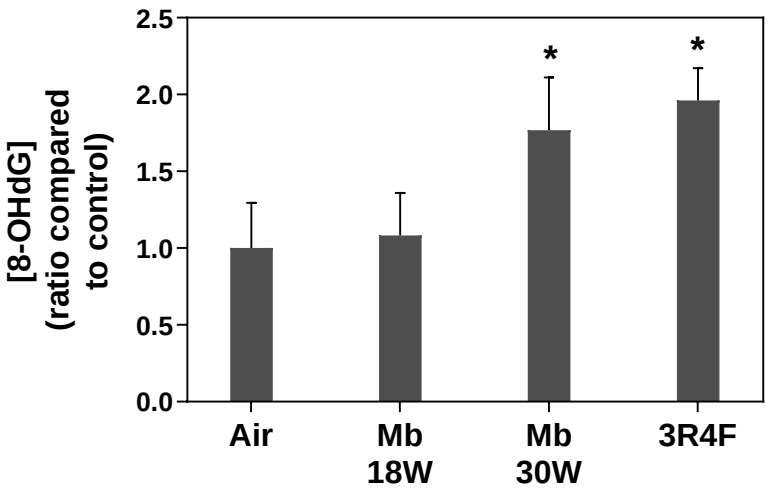
FIGURE 3


A
In vivo comet assay (liver)
% Tail Intensity
100
80
60
40
20
0
Air
Mb 18W
Mb 30W
3R4F
MMS
hOGG1 (-)
hOGG1 (+)
B
In vivo comet assay (lung)
% Tail Intensity
80
60
40
20
0
Air
Mb 18W
Mb 30W
3R4F
MMS
hOGG1 (-)
hOGG1 (+)
C
In vivo micronucleus test
MNPCE / 1000 cells
PCE / NCE
20
15
10
5
0
5
4
3
2
1
0
Air
Mb 18W
Mb 30W
3R4F
MMS
D
In vivo Pig-a gene mutation assay
Mutants x 10^{-6} cells
% RETs
120
20
15
10
5
0
8
6
4
2
0
Air
Mb 18W
Mb 30W
3R4F
ENU
RBC
RET

A


Subacute
[8-OHdG]
(ratio compared
to control)
2.0
1.5
1.0
0.5
0.0
*
*
*
Air
30
min
60
min
90
min
30
min
60
min
90
min
3R4F
Mb18W
Mb30W

B


Subchronic
[8-OHdG]
(ratio compared
to control)
2.5
2.0
1.5
1.0
0.5
0.0
*
*
Air
Mb
18W
Mb
30W
3R4F

C

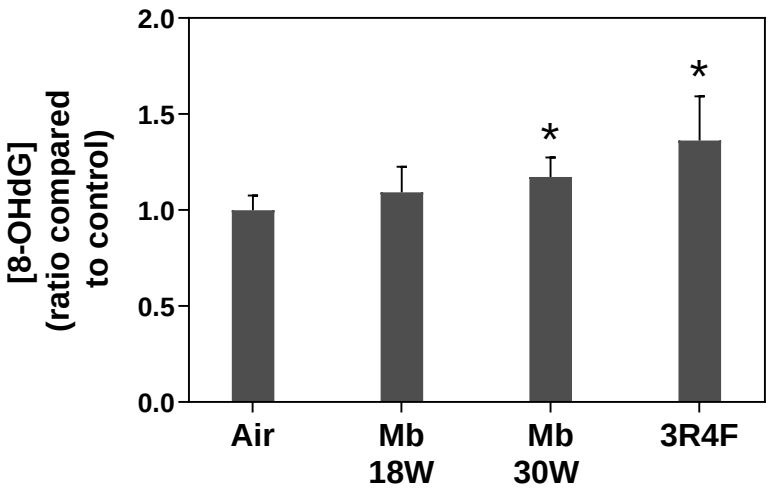

Chronic
[8-OHdG]
(ratio compared
to control)
2.0
1.5
1.0
0.5
0.0
*
*
Air
Mb
18W
Mb
30W
3R4F

TABLE 1

***In vivo* tests**	**End-points**	**Target organs**	**Exposure time**		
			4 days	**3 months**	**6 months**
Standard comet assay	Primary DNA damage	Liver, lung	X	X	X
Micronucleus test	Chromosomal aberrations	Bone marow		X	X
***Pig-a* gene mutation assay**	Gene mutations	Erythrocytes		X	X
hOGG1-modified comet assay	Oxidative DNA damage	Liver, lung			X
8-OHdG assay	8-OHdG	Lung	X	X	X

TABLE 2

	4 days			3 months			6 months		
	Mb 18W	Mb 30W	3R4F	Mb 18W	Mb 30W	3R4F	Mb 18W	Mb 30W	3R4F
Standard comet assay	-	-	-	-	-	-	-	+	+
Micronucleus test	n.a.	n.a.	n.a.	-	-	-	-	-	-
***Pig-a* assay**	n.a.	n.a.	n.a.	-	-	-	-	-	-
hOGG1-modified comet assay	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	-	+	+
8-OHdG assay	-	+	+	-	+	+	-	+	+

Graphical abstract

Cigarette

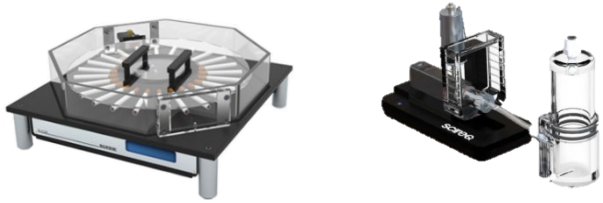
3R4F

E-cigarettes

Modbox
(18W and 30 W)

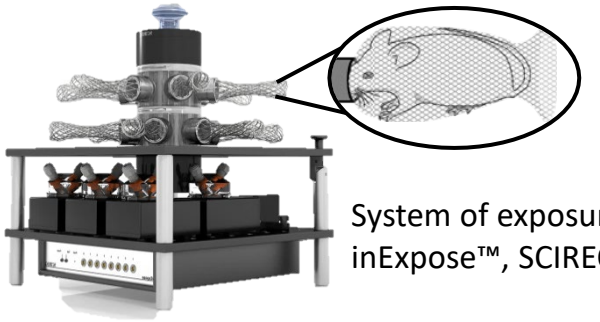
Smoking machine

Health Canada
Intense puff profile:
Volume : 55 mL
Duration : 2 s
Frequency : 2/min



Nose-only exposure

BABL/c mice



System of exposure :
inExpose™, SCIREQ

Exposure time

4 days 30, 60, 90 min/day

3 months 60 min/day, 5 days/week

6 months 60 min/day, 5 days/week

In vivo genotoxicity

- Standard comet assay
- hOGG1-modified comet assay
- Micronucleus test
- *Pig-a* test
- 8-OHdG assay

Results

3R4F and Modbox 30W induced DNA oxidative damage in lung and liver cells