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ORIGINAL ARTICLE

Electronic Cigarettes with Different Nicotine Concentrations in Unflavoured Liquid Induce Oxidative Stress

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Abstract

Background/Aim: Nicotine content and flavour in electronic cigarette (e-cig) liquids have been demonstrated to cause oxidative stress in acute exposure. However, the chronic effects of using unflavoured and with or without nicotine in e-cigs liquid have not been evaluated. This *in vivo* study aims to investigate the chronic effect of e-cig exposure with unflavoured liquids at different nicotine concentrations on oxidative stress.

Methods: The 24 male Wistar rats were divided into four groups of six each. Normal, as a control group. Nic 0, Nic 6 and Nic 12 groups were exposed to unflavoured e-cig liquid for eight weeks with different nicotine concentrations: 0, 6 and 12 mg/mL, respectively. E-cig exposure in rats was conducted using an exposure instrument adjusted to real-life exposure to humans. Oxidative stress markers, including plasma, liver and lung malondialdehyde (MDA) and superoxide dismutase (SOD), as well as plasma catalase (Cat) and glutathione peroxidase (GPx) were assessed at the end of the study.

Results: Unflavoured e-cig liquids induced oxidative stress in a nicotine concentration-dependent manner, in which the nicotine content of 12 mg/mL demonstrated the greatest response. There was a significant increase in plasma, liver and lung MDA and concurrently decreased plasma and selected organs SOD, as well as plasma Cat and GPx in all nicotine concentration exposed groups compared to the Normal group.

Conclusions: Chronic unflavoured liquids in e-cig exposure at different nicotine concentrations induced oxidative stress, potentially leading to various oxidative stress-induced diseases.

Key words: E-cig; Flavour; Liquid; Nicotine; Oxidative stress.

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Introduction

Electronic cigarette (e-cig) or electronic nicotine delivery systems (ENDS) is not considered an alternative to conventional cigarette only but are also used by people who have never smoked.[1] The fact that e-cigs are easily available in online stores, social media and vape stores at low prices results in a high prevalence of e-cig use among adolescents, 6.3 % in females and 29 % in males, as reported in an epidemiological study in Jakarta, Indonesia.[2] Furthermore, Indonesia has the worst tobacco control regulations compared to other Southeast Asian countries, with no specific regulations on e-cig control at the national and regional levels.[3] It is estimated that the e-cig usage trend will continue to increase yearly.

The use of a conventional cigarette and e-cig, both acute and chronic, has been reported in several previous studies to induce oxidative stress, leading to chronic obstructive pulmonary

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disease (COPD), hepatic steatosis and various diseases.[4–7] Accumulating evidence has shown that this effect is associated with nicotine content as an independent risk factor, either without or through the combustion process in e-cig and conventional cigarettes, causing oxidative stress and inflammatory responses.[5,8,9] Oxidative stress is a state of imbalance between reactive oxygen species (ROS) free radicals and antioxidants as detoxifiers of these reactive substances.[10] ROS generated by endogenous and exogenous sources, including conventional cigarettes and e-cig, contribute to deoxyribonucleic acid (DNA) damage.[11] However, the body has a defence mechanism to counteract oxidative stress through several antioxidant enzyme defences, including superoxide dismutase (SOD), catalase (Cat) and glutathione peroxidase (GPx).[10, 11] Previous studies reported that antioxidant activity decreased after e-cig exposure, indicating that the antioxidant defence mechanism was inadequate to counteract excessive oxidative stress.[12, 13]

Besides the nicotine content, e-cig aerosols produced during combustion contain harmful components, including acrolein acetaldehyde and formaldehyde.[14, 15] In addition, acute e-cig exposure with commercially available flavourings in liquids was confirmed in an *in vitro* study, playing a role in inflammation and oxidative stress even in the absence of nicotine.[12] A previous *in vivo* and *in vitro* study by Lerner et al[5] demonstrated that acute exposure to e-cig with flavourings and nicotine causes an increase in ROS, contributing to the inflammatory response and oxidative stress.

The chronic effects of using e-cig without flavours and with or without nicotine have not been evaluated and studies in experimental animals are limited. This study aimed to investigate the chronic effect of exposure to e-cig with unflavoured liquids at different nicotine concentrations *in vivo* on systemic, lung and hepatic oxidative stress characterised by malondialdehyde (MDA), SOD, Cat and GPx. It was hypothesised that all e-cig exposure groups with different nicotine concentrations without flavouring induce oxidative stress.

Methods

Study design

Using the resource equation formula, the sample size calculation for four groups resulted in 4-6 rats in each group.[16] Twenty-four male Wistar rats weighing 100 ± 20 g aged 4-5 weeks old were obtained from *Laboratorium Penelitian dan Pengujian Terpadu,* Universitas Gadjah Mada, Yogyakarta, Indonesia. The rats have housed two rats per cage at a 22 ± 2 °C temperature, 50 ± 10 % humidity and under a light/dark cycle of 12 h. Rats were given tap water and standard feed *ad libitum*. The rats were acclimatised for seven days before beginning of treatments, then randomly divided into four groups (n = 6 each):

1. Normal: a control group that did not receive any exposure,
2. Nic 0: exposure to 0 mg/mL nicotine concentration in e-cig liquid,
3. Nic 6: exposure to 6 mg/mL nicotine concentration in e-cig liquid,
4. Nic 12: exposure to 12 mg/mL nicotine concentration in e-cig liquid.

After eight weeks of e-cig exposure treatment, rats were fasted for 12 h and continued to be injected intramuscularly with 50-75 mg/kg of *Zoletil 50* (25 mg/mL tiletamine hydrochloride and 25 mg/mL zolazepam hydrochloride) supplied by *Virbac SA* (Carros, France) for general anaesthesia. Thus, 2 mL of blood from the heart was drawn and then put in EDTA tubes and the lung and liver were collected for biochemical assay. Before the study began, ethical approval was issued by the Ethics Committee of the Faculty of Medicine of Universitas Islam Indonesia, Yogyakarta, Indonesia (No 14/Ka.Kom.Et/70/KE/VIII/2021).

E-cig liquid formulation

E-cig liquid was adapted with modification as described previously by Glynos et al,[6] formulated by mixing propylene glycol (PG) (*Dow,* Jakarta, Indonesia), vegetable glycerine (VG) (*Iniko Karya Persada,* Jakarta, Indonesia) and pure nicotine liquid (*RTS Vapes,* NC, USA). To avoid study bias caused by flavour additives, the unflavoured e-cig liquid was used. The liquid was made in 100 mL for three different nicotine concentrations (0, 6 and 12 mg/mL) without flavouring agents, as follows: 1) Nicotine of 0 mg/mL liquid was formulated by mixing 40 % PG and 60 % VG; 2) Nicotine of 6 mg/mL liquid was formulated by mixing 34 % PG, 60 % VG and 6 % pure nicotine;

3) Nicotine of 12 mg/mL liquid was formulated by mixing 28 % PG, 60 % VG and 12 % pure nicotine (percentage equals volume in 100 mL liquid).

To monitor the stability of ingredients and e-cig liquid formulation over time, the standard method of storage recommended by the supplier was followed. The e-cig liquid was stored in a tightly closed glass container at room temperature and kept in the dark. The liquid was periodically checked for changes in colour, odour, viscosity and signs of separation or precipitation. The e-cig liquid remained stable over time and did not exhibit any significant changes in its physical properties or composition.

E-cig exposure instrument and protocol

A commercially available mod e-cig with batteries and atomisers was used (*Dovpo*, Guangdong, China). Exposure to e-cigs was carried out using the semi-automatic exposure instrument, as detailed information was reported in a previous study.[17] A group of exposed rats was placed in the exposure instrument chamber according to their respective group. They were exposed to 0, 6 and 12 mg/mL of nicotine concentration in e-cig liquid for 25 cycles (1 cycle consisting of a 5-second puff, 30-second interval and 30-second exhaust) per day, adjusting for real-life exposure to humans.

In preparation for the e-cig exposure in this study, a preliminary investigation on several e-cig users to gain insight into their typical usage patterns was conducted. Investigation showed that users generally inhale for 2-3 seconds, followed by an interval of 5-10 seconds to allow the aerosol to remain in the user's respiratory tract and followed by exhaling slowly. However, to adapt this to animal experiments, some adjustments were made. To ensure that semi-automatic exposure instrument delivered enough aerosol to the chamber within sufficient time, 5-second puffs with a 30-second interval were produced. As a group of rats was in the chamber, it was necessary to ensure that they inhaled the aerosol produced by the e-cigs and were assisted by the pump to flow slowly, mimicking human exposure. Therefore, the interval was set to 30 seconds. After exposure, the chamber was cleaned for 30 seconds using a mini fan and an exhaust air pump to eliminate any residual aerosol. Finally, the 25 cycles obtained from the time it took to spend one cigarette in previous study were used and replicated for e-cig use.[17]

Plasma and tissue preparation for assay

Collected blood samples were centrifuged at 1073 × g (at 4 °C) for 10 min to obtain plasma. Thus, plasma separated and transferred to microcentrifuge tubes and stored at -25 °C until analysis within less than a week.

Liver and lung organs were stored immediately after collection at -25 °C until ready for assays within less than a week. To prepare the tissue for MDA assay, 1 g of liver and lung tissues were homogenised with phosphate-buffered saline (0.01 M, pH 7.4) and centrifuged at 10,000 × g for 10 minutes (at 4 °C). The supernatant obtained was used for MDA assay.

For SOD assay tissue preparation, 1 g of liver and lung tissues were homogenised using a solution containing 0.1 M Tris/HCl (pH 7.4), 0.5 % Triton X-100, 5 mM β-ME and 0.1 mg/mL PMSF on ice. Centrifuge the resulting homogenate at 14,000 × g for 5 minutes (at 4 °C) to obtain supernatant for SOD assay.

Biochemical assay

MDA in plasma and liver and lung tissue supernatant assays were performed using commercial reagent kits and standard protocol provided by *Elabscience* (Wuhan, Hubei, China). The MDA assessment was carried out using the colourimetric method (Catalogue No: E-BC-K025-S) based on its reaction with thiobarbituric acid-reactive substances that produces a pink chromogen, then measured with a spectrophotometer at 532 nm.

A colourimetric method was also performed to assess plasma and tissue supernatant SOD activity (Catalogue No: K335-100) at 450 nm, plasma Cat activity (No: K773-100) at 570 nm and plasma GPx activity (No: K762-100) at 340 nm using a microplate reader according to manufacturer's standard protocols and their respective commercial reagent kits provided by *BioVision* (Milpitas, California, USA).

Plasma AST and ALT for assessing liver function were performed using an optimised UV-test method with a commercial reagent kit and standard protocol provided by *DiaSys* (Holzheim, Germany).

Statistical analysis

Data analysis was performed using IBM SPSS

26 (Chicago, IL, USA). Statistical analysis was determined using One-way ANOVA followed by Tukey's post hoc test for multiple comparisons. All data were displayed as the mean ± standard deviation (SD). The $p < 0.05$ was considered significant.

Results

Oxidative stress in plasma

Plasma MDA levels shown in Figure 1a indicated that e-cig exposure for eight weeks was significantly increased in e-cig-exposed groups compared to the Normal group. Plasma MDA level in the nicotine variation groups was highest in Nic 12, followed by Nic 6 and Nic 0, with significant differences among the exposed group.

Contrary, compared to the normal group, different nicotine concentrations demonstrated a significant decrease in SOD, GPx and Cat activities in e-cig-exposed groups, as shown in Figure 1b-d. The lowest mean of these antioxidant activities was found in Nic 12 group, followed by the Nic 6 and Nic 0 groups, with significant differences among them by post hoc analysis except SOD activity between Nic 0 and Nic 6 groups.

Oxidative stress and function in liver

Oxidative stress in the liver was induced, reflected by high levels of MDA significantly in the groups exposed to e-cigs with liquid containing nicotine content variations compared to the Normal control group. The Nic 12 group had the highest levels of MDA, followed by the Nic 6 and Nic 0 groups, with a significant difference among exposed groups (Figure 2a).

Regarding liver SOD activity, a significant decrease in all exposed groups compared to the Normal group was observed. The lowest plasma GPx activity was in Nic 12, followed by Nic 6 and Nic 0. In the exposed groups, no significant difference between Nic 0 and Nic 6 groups, but Nic 12 group had significant differences compared to Nic 0 and Nic 6 groups (Figure 2b). Liver function was impaired after exposure to e-cig, characterised by increased levels of AST and ALT in plasma in e-cigs exposed groups compared to the Normal group. The highest levels of both liver function markers were in Nic 12, followed by the Nic 6 and Nic 0 groups, with a significant difference among them (Figure 2c-d).

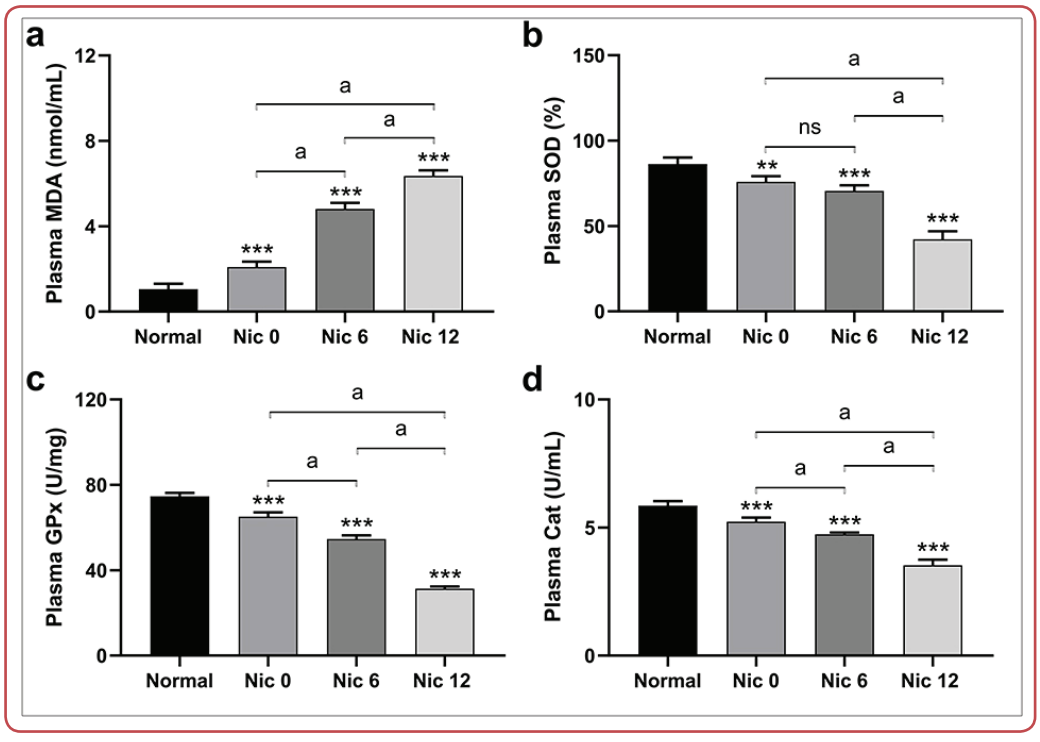

Figure 1: Plasma oxidative stress markers after e-cig exposure to different nicotine concentrations

****p < 0.001; **p < 0.01 compared to the Normal group; a = p < 0.001; ns = not significant; MDA: malondialdehyde; SOD: superoxide dismutase; GPx: glutathione peroxidase; Cat: catalase; Normal: a control group that did not receive any exposure; Nic 0: exposure to 0 mg/mL nicotine concentration in e-cig liquid; Nic 6: exposure to 6 mg/mL nicotine concentration in e-cig liquid; Nic 12: exposure to 12 mg/mL nicotine concentration in e-cig liquid.*

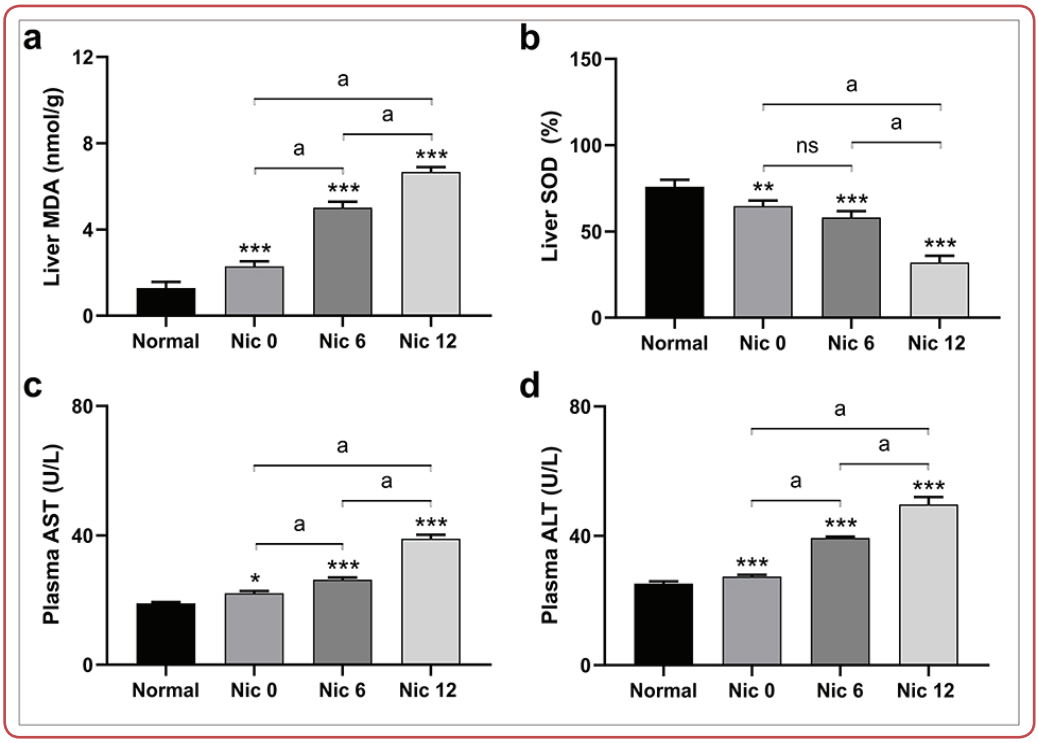

Figure 2: *Liver oxidative stress markers and function after e-cig exposure to different nicotine concentrations*

****p < 0.001; **p < 0.01 compared to the Normal group; a = p < 0.001; ns = not significant; MDA: malondialdehyde; SOD: superoxide dismutase; AST: aspartate aminotransferase; ALT: alanine aminotransferase; Normal: a control group that did not receive any exposure; Nic 0: exposure to 0 mg/mL nicotine concentration in e-cig liquid; Nic 6: exposure to 6 mg/mL nicotine concentration in e-cig liquid; Nic 12: exposure to 12 mg/mL nicotine concentration in e-cig liquid.*

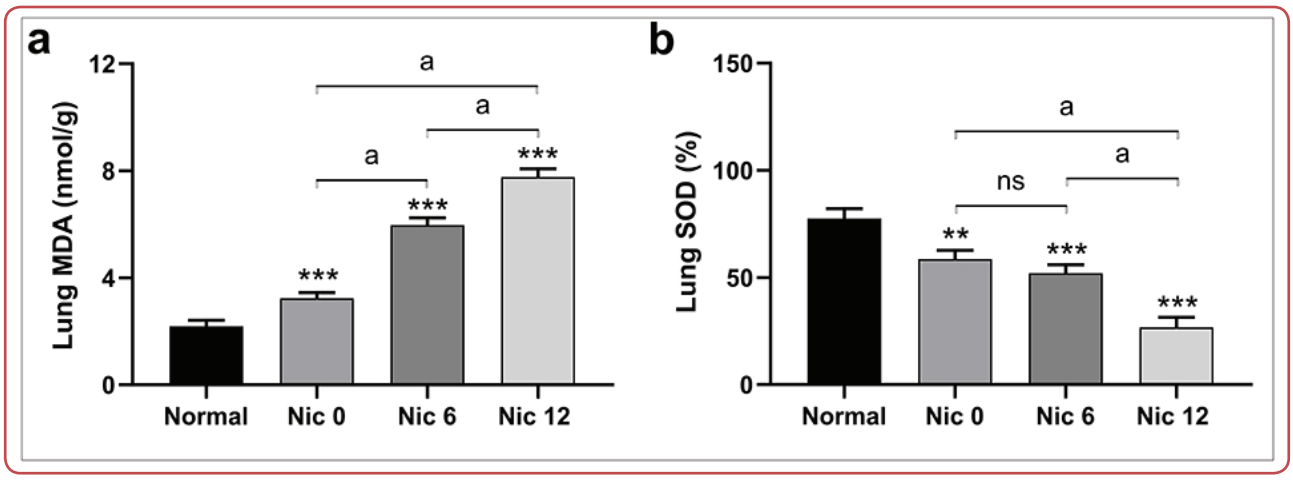

Figure 3: *Lung oxidative stress markers after e-cig exposure to different nicotine concentrations*

****p < 0.001; **p < 0.01; *p < 0.05 compared to the Normal group; a = p < 0.001; ns = not significant; MDA: malondialdehyde; SOD: superoxide dismutase; Normal: a control group that did not receive any exposure; Nic 0: exposure to 0 mg/mL nicotine concentration in e-cig liquid; Nic 6: exposure to 6 mg/mL nicotine concentration in e-cig liquid; Nic 12: exposure to 12 mg/mL nicotine concentration in e-cig liquid.*

Oxidative stress in the lung

Variation concentration of nicotine contained in e-cig liquid caused an increase in lung MDA levels compared to the Normal control. The highest dose of nicotine in the Nic 12 group had a higher impact on increasing lung MDA levels, followed by Nic 6 and Nic 0 groups. There was a significant difference among the exposed groups (Figure 3a).

The rats treated with different nicotine concentrations in e-cig liquid demonstrated significant results compared to the Normal group. Lung SOD activity was lowest at Nic 12, followed by Nic 6 and Nic 0, with significant differences between the exposed groups (Figure 3b).

Discussion

A previous *in vitro* study revealed that flavourings acetoin, pentanedione, diacetyl, cinnamaldehyde, ortho-vanillin, maltol and coumarin of e-cig liquid without nicotine induce an inflammatory response with an increase in interleukin (IL)-8 in human monocytes mediated by increased ROS production.[12] E-cig is also believed to be a safer alternative to conventional cigarettes. However, the potential chronic effects of its use are not clearly understood and study on experimental animals is still limited. Therefore, this study aimed to investigate the chronic effect of e-cig exposure to unflavoured liquid on the rat's model. The different nicotine concentration in e-cig liquid without flavours was observed. The hypothesis that different levels of nicotine content, including absence (0 mg/mL), mild (6 mg/mL) and high (12 mg/mL) can cause oxidative stress was confirmed. This is evidenced by increased levels of MDA, a reactive compound of lipid peroxidation, in the plasma, liver and lung. Additionally, a decrease in the activity of antioxidant enzymes such as SOD in the plasma, liver and lung, as well as Cat and GPx in the plasma was observed. Furthermore, oxidative stress revealed in a concentration-dependent manner that the higher nicotine concentration in e-cig liquids further exacerbates oxidative stress. In addition, this study also demonstrated that PG and VG without nicotine components in e-cig liquid cause oxidative stress.

The process of generating oxidative stress either from e-cig or liquid is not fully understood. However, a previous study confirmed the formation of OX/ROS in e-cig for two reasons. Firstly, due to the activation of the liquidless heating element, replacing the new heating element has the highest OX/ROS generation compared to multi-use and when the heater is activated it produces a small amount of aerosol without the addition of liquid. Second, through the e-cig liquid vaporising process, a drop of liquid causes a fluorescence spike in oxidised dichlorofluorescein using a multi-use heat element on the fourth use.[5]

It has been reported that without the combustion process in e-cig and conventional cigarettes nicotine administration induce oxidative stress. Subcutaneous injection of nicotine alone to rats for a month has been confirmed to induce oxidative stress in a concentration-dependent manner, as reported in the previous study, which increased MDA and decreased total antioxidant capacity.[9] Simultaneously, the mean of ZO-1 immuno-positive cells decreased and vascular endothelial growth factor increased in the group with higher nicotine concentrations, indicating dysfunction of the airway epithelial barrier and increased angiogenesis.[9, 18] In another *in vivo* study, two months of intraperitoneal nicotine injection caused oxidative stress by mediating increased MDA, decreased activity of SOD, Cat, reduced glutathione (GSH), oxidised glutathione (GSSG), GPx and glutathione transferase (GST). Furthermore, an excessive inflammatory response was also observed with increased tumour necrosis factor (TNF)-α, IL-17 and nuclear factor (NF)-κB expression.[13] These findings suggest that nicotine alone independently induces oxidative stress and inflammation without combustion in e-cig and conventional cigarettes.

The current study demonstrated increased plasma, liver and lung MDA levels in all groups of rats exposed to an e-cig aerosol with different nicotine concentrations corresponding to increased nicotine concentration. AST and ALT levels also increased, indicating oxidative stress-induced liver damage. Simultaneously, the activity of plasma, liver and lung SOD, as well as plasma Cat and GPx, decreased, which indicates oxidative stress generated by chronic e-cig aerosol exposure. That is in line with a previous *in vitro* study by Lerner et al[5] that demonstrated that e-cig aerosol exposure in human airway epithelial cells (H292) increased IL-6 and IL-8 expression. Furthermore, exposure to e-cig aerosol in C57BL/6J mice increased monocyte chemoattractant protein (MCP)-1, IL-1α, IL-6 and IL-13 expression compared to the control group. A previous *in vivo* study reported that NOX-2-mediated chronic e-cig exposure induces oxidative stress that develops vascular, brain and lung damage.[8] In addition, e-cig use induces hepatic steatosis through increased oxidative stress and hepatocellular apoptosis, as well as disruptions in cholesterol and lipid metabolism and the liver's circadian system.[7] Another study reported that acute e-cig exposure induces inflammation and oxidative stress, resulting in cognitive impairment.[19] Exposure to e-cig aerosol, both with and without nicotine, has been shown to induce oxidative stress by enhancing the inflammatory response, indicating that other constituents of e-cig liquids besides nicotine may also be involved.

Previous studies have reported the effect of nicotine on e-cig without distinguishing whether the parameter changes are because of the nicotine, the flavours or PG and VG as the main component of e-cig liquid.[6, 20] When heated at high temperatures, PG and VG lead to carbonyl compounds formation, such as acrolein, acetaldehyde and formaldehyde.[21, 22] Carbonyl compounds have irritant properties and cause acute inhalational toxicity. Acrolein exposure has been known to induce ROS generation, cause pulmonary inflammation associated with COPD, contribute to asthma and induce p53 adduction in the development of lung cancer.[23] An *in vitro* study by Chen et al[24] in human lung epithelial BEAS-2B cells demonstrated that acetaldehyde exposure reduced lysine residues acetylation at the N-terminus of cytosolic histones H3 and H4 and could inhibit chromatin assembly. These findings indicate that defective chromatin assembly is associated with dysregulation of transcription, DNA replication and repair and genome instability. Formaldehyde also causes airway irritation and impairs pulmonary function, which is associated with asthma.[25] Meanwhile, PG and VG in e-cig liquid exposure for four weeks in mice increased bronchoalveolar lavage fluid (BALF) cellularity, induced oxidative stress, increased epithelial Muc5a production and negatively impacted lung mechanics suggesting respiratory irritation.[6] In line with presented study, exposure to e-cig aerosol with liquid containing PG and VG without nicotine causes an increase in oxidative markers in rats.

Conclusion

In summary, oxidative stress plays an essential and broad role in pathogenesis in many organ systems. This study suggests that chronic exposure to e-cig with unflavoured liquids, with or without nicotine, induces systemic as well as liver and lung organs oxidative stress in a concentration-dependent manner, which in turn, potentially leads to a variety of oxidative stress-induced diseases.

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Conflict of interest

The authors declare there is no conflict of interest. This study does not get financial support from any conventional cigarette or e-cig company.

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