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Research Article

Impact of Nicotine and Nicotine-Free E-Cigarettes on Body Weight, Erythrocyte Dynamics, Sperm Morphology, and Molecular Outcomes in Mice

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ABSTRACT

The effect of electric cigarettes (e-cigs) on health should be a concern since their legality has occurred in Indonesia. Young adults use e-cigarettes not as therapy but as a lifestyle. The vapor component contains propylene glycol, vegetable glycerin, flavor, and nicotine, which may harm human health. This study examined the health risks of e-cigarettes on body weight, erythrocytes, and sperm morphology in mice. We used twenty healthy male mice exposed to the cigarette's vapor for thirty days. Mice were divided into room air as the negative control, nicotine-free, and 18 mg nicotine-containing groups. Our results showed that the body weight of the nicotine-free and nicotine-containing groups increased, while micronuclei formation increased after e-cig exposure. Furthermore, abnormal sperm morphology in the nicotine-containing groups increased significantly, yet the nicotine-free group was not significantly different compared to the negative control. These results were supported by the molecular prediction that exposure to e-cigs might increase oxidative stress and decrease the expression of TGF-β. Moreover, studies on it are ongoing to gather scientific evidence.

1. Introduction

Electric cigarettes (e-cigs) are an alternative to tobacco cigarettes using a tool with batteries to deliver liquid materials, such as nicotine, flavor, and other chemical substances, combusted by a heating element, named an atomizer, resulting in vapor inhaled by smokers. The World Health Organization (WHO) defines e-cigarettes as electronic Nicotine Delivery Systems, which are also known by various names, including re-juice, vape, and smart cigarettes (Organization 2020; Shellasih *et al.* 2022). The sales of e-cigarettes in Indonesia increased significantly from 2015 to 2019, and at an annual rate, it is predicted to persist at a higher level in 2023 (Shellasih *et al.* 2022). At the start, e-cigs were provided for therapeutic purposes to people with tobacco smoke addiction since the amount of nicotine in e-cigs can be set. However, e-cigs have recently become a lifestyle among Indonesians since the market regulation for controlling e-cig markets (van der Eijk *et al.* 2022).

Southeast Asian countries, such as Singapore, Cambodia, Laos, Brunei, and Thailand, have strict prohibitions on the sale, import, and use of e-cigarettes. Other countries, including the Philippines and Malaysia, restrict e-cigs, such as permitting e-cigs with a particular restriction, for example, the constraint on nels in Malaysia. Advertising controls regulate advertisement copy, targeting youths, and clothing for youths, as well as the use of e-cigarettes in public places in the Philippines and Vietnam. Several provinces in Malaysia (Penang, Kelantan, Terengganu, Johor, and Kedah) have already

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banned e-cig sales. Meanwhile, Myanmar and Indonesia still have no specific regulations (van der Eijk *et al.* 2022). There is no doubt that in Indonesia, between May and June 2022, there were 139 large e-cig store retailers, with potentially more stores in other cities, and approximately 5,000 retailers (Shellasih *et al.* 2022). Mass advertisements about e-cigs from influencers, especially in Indonesia, cause the number of e-cig users to rapidly increase. Still from the same sources, Indonesians use e-cigs because of the trend and still consider them unharmful (Shellasih *et al.* 2022).

Since e-cigs are still considered harmless and have no regulation, especially among Indonesians, it is quite challenging to find the number of accidents or health problems caused by e-cigs. Reports have surfaced of numerous incidents, including explosions from e-cigarettes, burns on the chest, eyelids, and fingers, and irritations on the upper respiratory tract, nose, and eyes (van der Eijk *et al.* 2021). E-cigs and their chemical components are most likely responsible for these incidents. Components in electronic cigarettes that are suspected to be hazardous include nicotine, formaldehyde, propylene glycol, nitrosamines, sulfur, and other toxic substances such as heavy metals and particulate matter. According to the study, all e-cigarettes supplied in Indonesia emit formaldehyde. The combustion process, which lasts for 60 minutes, results in the production of around 0.0345-0.1490 parts per million (ppm) of formaldehyde. Diacetyl is another toxic compound found in flavors. Diacetyl is a chemical molecule used as an additive in electronic cigarette products to produce specific flavors and scents, such as butter or caramel fragrances. Laboratory analysis identified diacetyl in 39 out of the 51 samples examined (Allen *et al.* 2016). According to the 2017 United States National Institute for Occupational Safety and Health report, continuous inhalation of formaldehyde and diacetyl over extended periods can pose significant health risks.

Our previous research showed that nicotine-free e-cigarettes decreased the concentration of sperm in mice by 42.56% compared to the control group (Mandasari *et al.* 2019). Undoubtedly, the presence of reactive components, such as propylene glycol (PG) and vegetable glycerin (VG), in the vapor is responsible for enhanced oxidative stress in preclinical experiments, as well as *ex vivo* and *in vivo* (Kaisar *et al.* 2017). These reactive chemicals have been observed to induce irritation in both mice and humans (Kienhuis *et al.* 2015). Additionally, they could form aldehydes, such as formaldehyde, benzaldehyde, and acrolein, during the heating process. Acrolein is a carcinogen that increases DNA adducts from buccal swabs of electric cigarette users (Cheng *et al.* 2022). Exposure to e-cigarettes over a 4-week period resulted in increased micronuclei formation in the tracheal tissue of rats, as observed under scanning electron microscopy (Cirillo *et al.* 2019). A study using 18-mg nicotine-containing e-cigarettes can also induce DNA damage by increasing the production of 8-hydroxy-2'-deoxyguanosine levels in the lung (Canistro *et al.* 2017).

However, the previous studies could not fully elucidate the impact of e-cigs since e-cig smokers are also dual smokers, which can confuse the e-cigs' impact itself (Azagba *et al.* 2019). This research aims to evaluate whether nicotine-free and nicotine-containing e-cigarettes have the potential to be harmful in the mouse model. The erythrocyte parameter is used because it plays a significant role in distributing oxygen and nutrients to cells. Erythrocytes exhibiting micronuclei indicate that the repair mechanism in the cells is compromised, resulting from chromosomal damage or interference during mitosis, which may contribute to cancer development (Bolognesi *et al.* 2013). Furthermore, as in tobacco smoking, fertility is still a matter, and evaluating this parameter might give a valuable output for e-cigarette users in the future. We also evaluate the body weight of mice to investigate the prevention of weight gain caused by e-cigarettes related to tobacco smoking cessation. Since chemical compounds in e-cigarettes are toxic and carcinogenic, we predict the genes and molecular pathways affected to support our results.

2. Materials and Methods

2.1. Animal Preparation and Condition

The Animal Care and Use Committee (ACUC) of the Faculty of Veterinary Medicine, Universitas Airlangga, Indonesia, approved the experiment protocol (No. 2.KE.130.07.2018). Twenty healthy male mice were purchased from the Animal Laboratory at the Institute of Tropical Diseases (ITD), Universitas Airlangga, Indonesia, at 3 months of age, with an average body weight of 25–30 g. Mice were bred in the Animal Care Laboratory at the Faculty of Health Sciences, Universitas Maarif Hasyim Latif, Indonesia and randomly distributed over four groups: room air group as negative control (C), positive control group (C+), nicotine-free group (G1),

and 18-mg nicotine group (G2); each consisting five mice. They were kept in a cage under a 12-hour light/12-hour dark cycle at 22°C, 60% humidity, and were fed ad libitum for a week before treatment. The C+ was given cyclophosphamide by intraperitoneal injection. Animals were weighed before and after each week for one month of treatment.

2.2. Exposure to E-cigarettes

The exposure was performed on the entire body of mice, using a 50 mL syringe for administering the vapor. The VGOD Pro Mech 2 Kit and Koffie Creme Salt Nicotine (0 mg and 18 mg) were purchased from a local store in Indonesia. The exposure was carried out for thirty days in a 28 cm × 20 cm × 15 cm propylene box. Exposure was tested three times, with 20 suction cycles followed by a 10-minute pause. The smoke of e-cigarettes was carried out using a three-way hole. The first hole was connected to the mouth of an e-cigarette, and the second hole was connected to a syringe. The hole was placed on the slightly opened box lid, while the 50 mL syringe hole connected the other end of the hole. The box lid was slightly opened, to which the third hole was placed. When the suction of the cigarette's smoke with a syringe worked, the third hole was closed, then opened, and the syringe was pushed to emit smoke.

2.3. Micronuclei Evaluation

Mice were anesthetized with 0.1 mg ketamine by intraperitoneal injection after thirty days of exposure. Blood samples from the tail vein were taken using an 18-G needle syringe and smeared onto an object glass. Ten slides per mouse were prepared and air-dried. Methanol was used to fix the cells, and they were stained using 1% Giemsa for 30 minutes, followed by rinsing under tap water. Micronuclei were examined using a light microscope with 400x magnification and then counted per 1,000 erythrocytes using Image RasterTM version 2.1.

2.4. Sperm Analysis

Mice were dissected using a sharp scalpel. Tests were carried out on a petri dish containing phosphate-buffered saline for removing blood. The epididymis, along with surrounding fat tissue, was added to 1% Sodium Chloride Solution (0.9% NaCl). One drop of sperm suspension was smeared onto a clean and free-greased object glass. Eosin Y was used for staining sperm.

2.5. *In-silico* Prediction of Molecular Gene Expression by Chemical Composition in E-cigarettes and Its Pathway

In this study, the main ingredient in e-cigarettes, propylene glycol (PubChem CID 1030), will be analyzed. The evaluation will also include benzaldehyde (PubChem CID 240) and acrolein (PubChem CID 7847), which are byproducts of e-cigarette combustion. The mRNA expression of all these chemical compositions will be investigated using DIGEP-Pred. Down-regulated and upregulated mRNA expression will be examined in further detail using the Enrichr tool. This analysis will identify the specific molecular pathways involved and enable predictions to be made about the impacts of e-cigarettes' chemical ingredients.

2.6. Statistical Analysis

All results were expressed as mean±SEM and analyzed by GraphPad Prism version 8 software, including graphs and statistical analysis. The data on micronuclei and sperm were analyzed using Ordinary One-Way ANOVA, and the Tukey Test—a Repeated Measures Two-Way ANOVA was used for the mice's body weight.

3. Results

3.1. Effect of Nicotine-free and Nicotine-containing E-cigarettes on the Body Weight of Mice

Both groups, G1 and G2, exposed to e-cigarettes, exhibited a decrease in body weight compared to the control group, as depicted in Figure 1. The G1 and G2 exhibited significant reduction compared to the C (each p-value < 0.0001). Additionally, the G2 showed a significant decrease compared to the G1 (p-value = 0.0021).

3.2. Micronuclei Formation after Nicotine-Free and Nicotine-Containing E-Cigarette Exposure

According to the findings presented in Figure 2, it is evident that exposure to e-cigarettes containing 18 mg of nicotine, as well as nicotine-free e-cigarettes, resulted in a notable increase in the development of micronuclei compared to the negative control (p = 0.0073 and 0.0121, respectively). Additionally, the group that was not exposed to nicotine (G1) had a

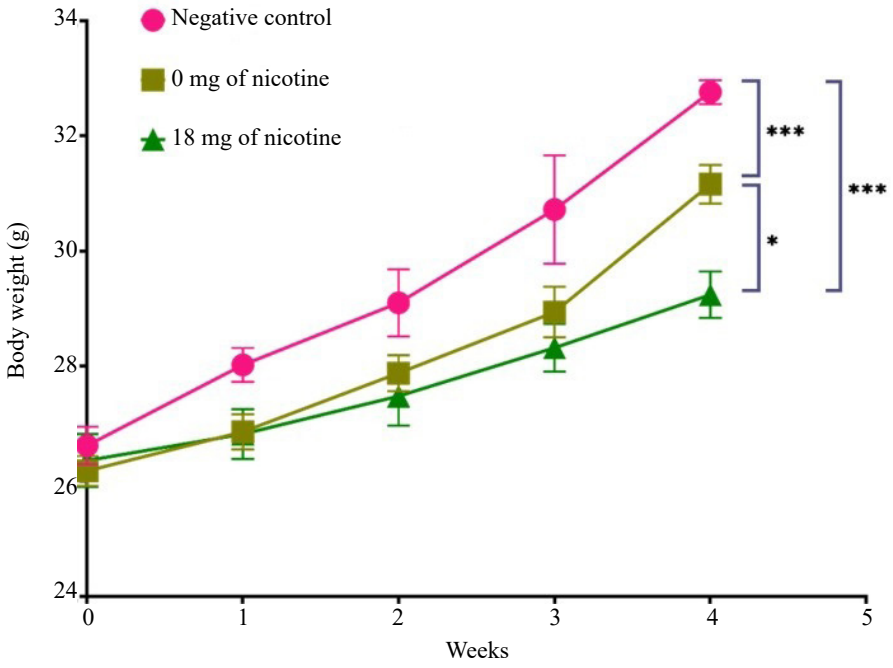

Figure 1. The body weight of the mice in each group at the end of experiments. The body weight of each group was monitored at weekly intervals for 4 weeks of exposure and depicted in the form of a line graph. The graph illustrates a significant decline in the e-cigarette treated groups in comparison to the negative control

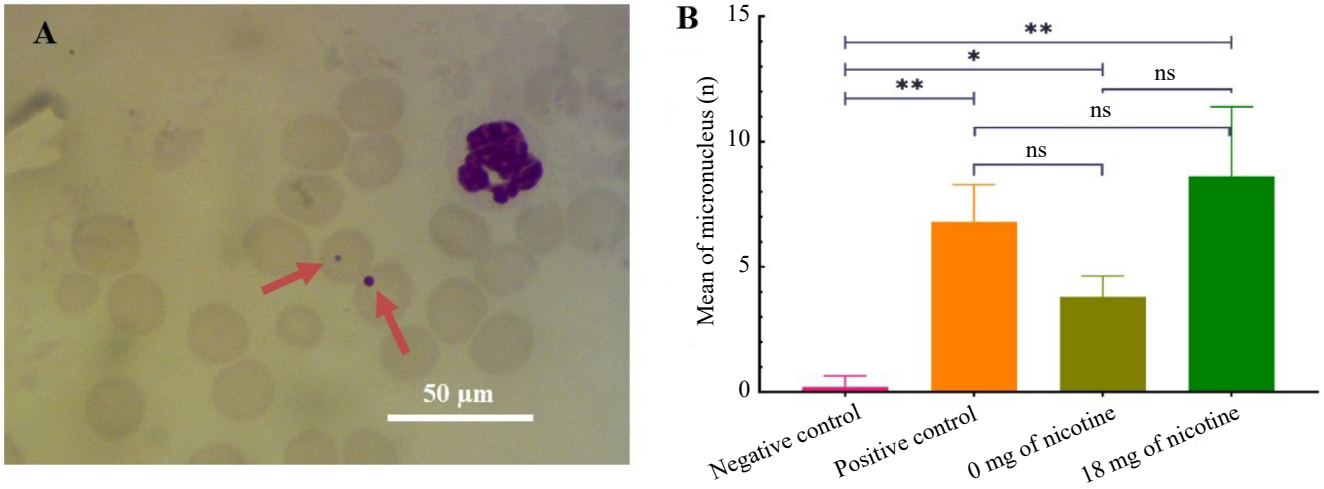

Figure 2. Micronuclei formation in erythrocytes. (A) The presence of micronuclei in erythrocytes occurred in both groups exposed to vapor (Giemsa, 400X). (B) The bar graph illustrates the mean values of micronuclei observed within each group (*/**: significance results; ns: non-significant)

similar lack of significant difference when compared to the group that was exposed to nicotine (G2). The G2 exhibited the largest number of micronuclei compared to all other groups.

3.3. Evaluation of the Effect of E-Cigarettes on Sperm Morphology

The findings of our study indicate a statistically significant increase in the prevalence of aberrant sperm in the group exposed to nicotine compared to the negative control group exposed to room air ($p = 0.0219$). On the other hand, exposure to vapor without nicotine led to a rise in aberrant sperm count, but this increase did not reach statistical significance. The present investigation documented various atypical sperm morphologies, including folded tails, heads lacking tails, heads lacking hooks, amorphous shapes, small heads, and sperm with no heads (Figure 3A).

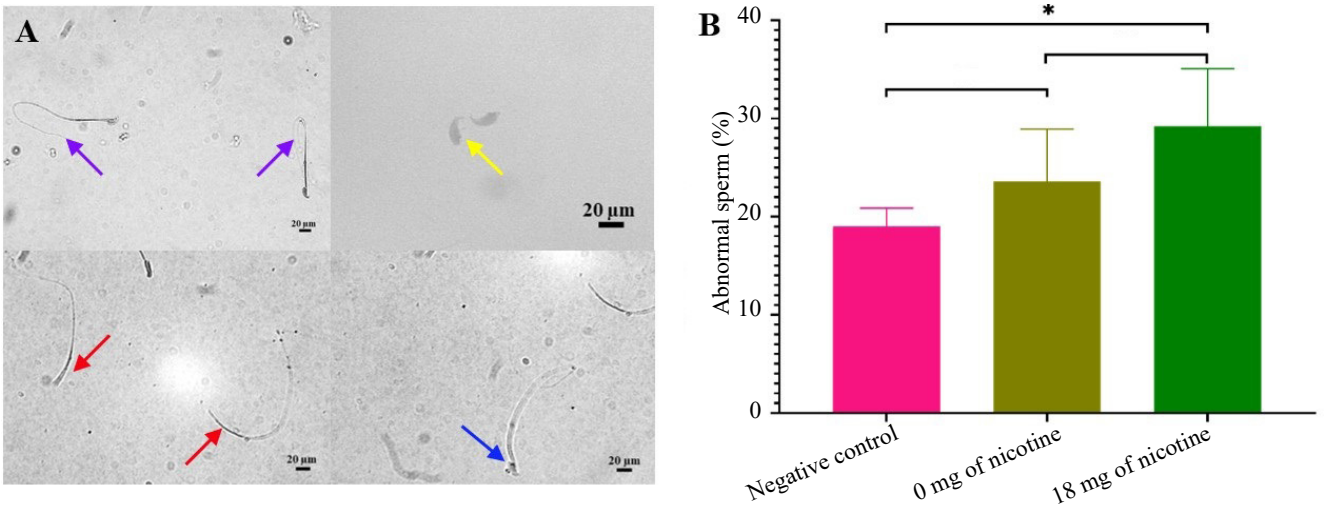

Figure 3. The morphology of aberrant spermatozoa was examined following a one-month period of exposure to vapor. (A) Various morphological abnormalities were observed in the sperm shown by different color arrows, including folded tails (purple), tailless heads (yellow), headless tails (red), and an amorphous head (blue) (Eosin Y, 400X). (B) The presented bar graph depicts the percentage of atypical sperm in various groups (*: significance results; ns: non-significant)

3.4. Down-regulation of mRNA Expression Affected by E-Cigarettes and Its Pathway

The prediction results showed that thirty-one mRNA expressions were down-regulated by propylene glycol, ninety genes were down-regulated by benzaldehyde, and thirty genes were affected by acrolein. All ingredients of e-cigarettes predictably decreased 13 genes, including SMARCC1, ABCC5, TMEM59L, IKBKAP, ABCA13, ELAVL1, ABCC13, PPP1CA, MFAP4, ERCC2, RARB, SYMPK, and H1FX (Figure 4A, Supplementary Data 1). Furthermore, in silico analysis reveals that these genes have strong p-values, which decrease the signaling of the TGF-β receptor complex across all compositions (Figure 4B).

3.5. Up-regulation of mRNA Expression Affected by E-Cigarettes and Its Pathway

We predicted that propylene glycol increased twenty-four mRNA expressions, eighty-eight genes increased by benzaldehyde, and twenty-four genes upregulated by acrolein. Propylene glycol, benzaldehyde, and acrolein are predicted to affect 11 genes with mRNA upregulation expression, including VNN1, GCLC, POR, FECH, HSPA1L, TXNRD2, KRT34, AKR1B10, SOD3, FTL, and GCLM (Figure 5A, Supplementary Data 2). The in silico molecular pathway analysis indicated that e-cigarettes increased the expression of genes involved in cellular stress response and ROS activity (Figure 5B).

4. Discussion

Tobacco smoking is well-known for leading to many diseases, such as chronic obstructive pulmonary disease (COPD), atherosclerotic diseases, and lung cancer (Kotlyarov 2023; Lu *et al.* 2024). It would be beneficial to quit smoking to prevent diseases. E-cigs with or without nicotine are used to replace tobacco smoking, which is considered safer. However, problems with tobacco smoking cessation may reduce physical activity and alter dietary behaviours, causing excessive weight gain that leads to obesity and type 2 diabetes (Bush *et al.* 2016; Vogel & Ramo 2021). It is noteworthy that nicotine has an anorexic effect, causing decreased appetite through two mechanisms. First, activating the melanocortin system plays a crucial role in suppressing appetite, resulting in reduced food intake. Second, nicotine causes an increase in the metabolic rate. Additionally, nicotine can modify the perception of a bitter taste, effectively masking the taste of food (Salamanca *et al.* 2018).

Our results found that treatment in the nicotine-free group (G1) could prevent body weight gain as well as in the nicotine-containing group (G2). An interview and observational study mentioned that 45 of 60 participants using nicotine e-cigs helped them to control their weight after smoking cessation, and 61 of 62 participants using flavored e-cigs because they could reduce food cravings (Kechter *et al.* 2022). A study by Yin *et al.* of 26 non-smoking female participants

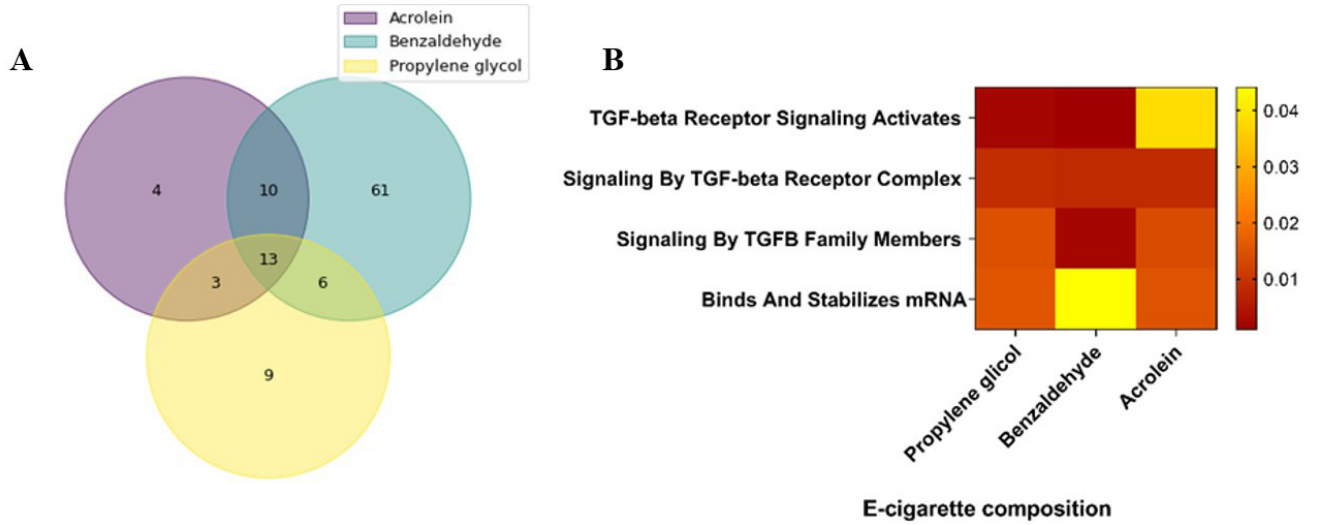

Figure 4. The prediction results of down-regulated genes affected by e-cigarette. (A) Thirty-one down-regulated mRNA expressions by propylene glycol, 90 by benzaldehyde, and 30 by acrolein. (B) Several genes have strong p-values to decrease signaling TGF-β receptor complex

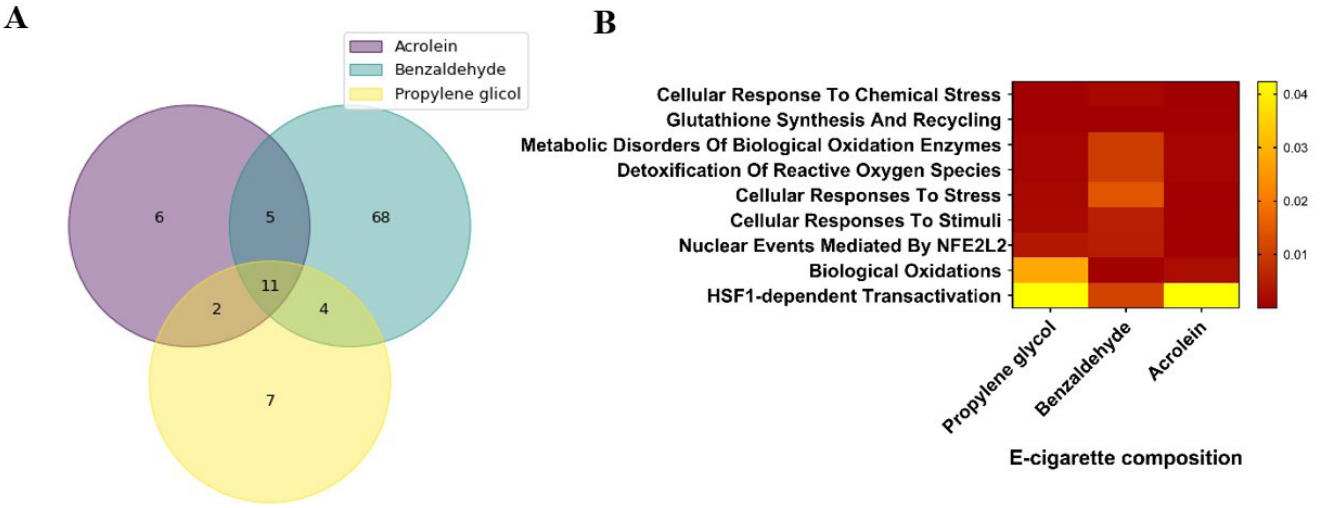

Figure 5. The prediction results of upregulated genes affected by e-cigarettes. (A) Twenty-four upregulated mRNA expressions by propylene glycol, 88 by benzaldehyde, and 24 by acrolein. (B) Several genes have strong p-values to increase oxidative stress

demonstrated that aroma and taste, either independently or in combination, reduced hunger sensation (Yin *et al.* 2017). Later, a study of a group of students aged 13 to 19 found that 13.8% of teenagers reported using flavoured e-liquids to control their appetite, while 9.3% used them for weight loss (Morean & Wedel 2017).

In addition, the use of flavoured e-liquids for either of these purposes was correlated with a higher frequency of vaping and greater consumption of flavoured e-liquids. Specifically, e-liquids with flavors like confectionery were linked to the use of vaping as a means of regulating appetite (Morean & Wedel 2017). A possible reason to explain this fact is that the taste and aroma of e-cigarettes could reduce appetite, decreasing food intake. The aroma was detected by the olfactory epithelium through both orthonasal and retronasal pathways, inducing neurons in the orbitofrontal cortex (OFC) to modulate hunger and satiety (Ruijschop *et al.* 2008; Hayes & Baker 2022). Identifying brain mapping studies revealed that the OFC is associated with appetite or satiety hormones, including peptide tyrosine-tyrosine (PYY) and ghrelin (Althubeati *et al.* 2022). PYY, through proopiomelanocortin neurons resulting in melanocortin four receptor inhibition, triggers the OFC to transmit a satiety signal (Mangan *et al.* 2019). Nevertheless, overweight and obesity caused

by smoking cessation might be prevented by using e-cigarettes with or without nicotine, yet their effect on health needs to be further discussed.

Based on our data, nicotine-free and nicotine-containing e-cigarette exposure induced higher micronuclei in erythrocytes than the negative control. Four weeks of exposure to nicotine-free (G1) as well as nicotine-containing e-cigarettes (G2) can lead to micronuclei formation. A study on epithelial cell lines revealed that e-cigs with or without nicotine exposure could significantly increase micronuclei formation (Tellez *et al.* 2021), supporting our results. Additionally, the combustion of e-cigs emits toxic trats's's such as formaldehyde. In another study, formaldehyde exposure trats's' whole body significantly increased the micronucleated polychromatic erythrocytes in both sexes (K *et al.* 2024).

Micronuclei are a genotoxicity parameter that indicates DNA damage in cells caused by chemical compounds, medicine, or food. In repairing mechanisms, ruptured micronuclei should be dismissed by the autophagy-lysosomal pathway. However, micronuclei may trigger an alternative pathway, including the cGAS-cGAMP pathway and cellular immune response, leading to inflammation that contributes to cancer metastasis (Björndahl and Brown 2022). In addition, the degradation of micronuclei has been slowly observed regarding their persistence in multiple cell cycles, resulting in DNA damage and instability that lead to chromothripsis, an initial step in cancer development (Reimann *et al.* 2023). Even though the observed frequency of micronuclei per 1,000 erythrocytes in our findings is generally low due to the elimination of micronuclei erythrocytes by the spleen, this research provides valuable insight. It reveals that the formation of micronuclei by e-cigarette exposure is undeniable, causing genomic instability during cell division, and that cellular repair becomes less effective. Infertility is a male health problem that is believed to be caused by tobacco smoking. According to the World Health Organization, abnormal morphology of sperm can affect infertility if there are more than 96% of sperm with abnormal morphology, known as teratozoospermia (Björndahl & Kirkman Brown 2022). Teratozoospermia is a condition in which sperm have excessive cytoplasmic enzymes caused by spermatogenesis malfunction through promoting endogenous ROS that might be mediated by the enzyme glucose-6-phosphate dehydrogenase (G6PD), resulting in the activation of nicotinamide adenine dinucleotide phosphate (NADPH) (Asadi *et al.* 2021). Another pathway, when cellular stressors or toxic materials from e-cigs activate the PIEKA-FYN complex, might induce phosphorylated STAT3, leading to its translocation to the nucleus, where it binds to the promoter to enhance transcription of the g6pd gene (Meng *et al.* 2022).

There is still a lack of studies on the effect of electric cigarettes on sperm. Our results show that there was no significant increase in abnormal morphology sperm in the nicotine-free group compared to the room air group; meanwhile, abnormal sperm increased significantly in the nicotine-containing group. There were 23.6% (236/1,000) abnormal sperm in the nicotine-free group and 29.2% (292/1,000) in the nicotine-containing group. In contrast to the results of Rahali *et al.* (2018), liquid electric cigarettes significantly increase abnormal sperm by more than 90% (Rahali *et al.* 2018). We suppose that the route of administration of liquid vapor, using intraperitoneal injection, should be considered and may affect the result. Although sperm parameters indicate that nicotine-free vapor is considerably safer than nicotine-containing vapor, this finding still reveals that vapor has potential harmful risks in long-term exposure, which warrants further investigation.

Regulation at several levels is crucial in signaling networks to ensure that the pathways function properly and carry out their normal physiological function. The transforming growth factor β (TGF-β) family is a group of growth factors and cytokines that are produced and have significant roles in various cellular processes, development, and diseases, including cancer. TGF-β exerts a potent inhibitory effect on the proliferation of various cell types, including epithelial, endothelial, hematopoietic, and immunological cells (Zhang *et al.* 2017; Massagué & Sheppard 2023). The TGF-β family proteins have a significant impact on cell differentiation, as they regulate the differentiation of all cell lineages at various stages of development (Derynck & Budi 2019; Massagué & Sheppard 2023).

In addition to cell proliferation and differentiation, TGF-β also performs various other biological tasks. These include promoting or protecting against cell death, enhancing the production of extracellular matrix (ECM) proteins, facilitating cell motility and invasion, and regulating cell metabolism (Derynck & Budi 2019). According to our prediction results, the chemical substances produced by the combustion of e-cigarettes may lead to a decrease in TGF-β signaling. As a result, this dysfunction is linked to the beginning or the advanced phases of certain diseases, such as fibrotic

conditions, long-term inflammatory disorders, and malignancies (Massagué & Sheppard 2023). Although this study did not investigate chronic conditions, these results suggest that the repair system in cells, including both micronucleated erythrocytes and abnormal sperm, was perturbed, as the TGF-β, which has homeostatic and repair functions, was decreased based on the molecular prediction test.

Reactive oxygen species (ROS) refer to a collection of extremely reactive molecules produced from oxygen. Physiological systems have both ROS generation and antioxidant defenses, which protect cells f "o" excessive and h" r" ful amounts of ROS. Oxidative stress, also known as "oxidative distress", refers to a significant imbalance between the production of ROS and the body's ability to counteract them with antioxidants (Silvestrini & Mancini 2024). As ROS are highly reactive, they can react with almost any large molecule, triggering lipid peroxidation and DNA damage, including DNA strand breaks and alterations in repair processes that can cause genomic alterations (Song *et al.* 2024). ROS can interfere with normal cellular function, generating perpetual cycles that lead to an increase in ROS. In another study, acrolein, one of the toxic substances produced during e-cig combustion, promoted a decrease in the non-enzymatic antioxidant in erythrocytes (Kopera *et al.* 2024), resulting in a higher ROS level in the cell. Innate immune cells, for example, neutrophils in a homeostatic environment, produce ROS to signal an inflammatory response, which in turn enables them to engulf damaged cells and pathogens (Liu *et al.* 2023). However, the ROS accumulation eventually results in opposite action, including increased oxidative damage to cells, altering macromolecules, and even the cell structure and function by interfering with several molecular pathways, such as mitogen-activated protein kinase (MAPKs) and phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt) (Afzal *et al.* 2023). A previous study revealed that e-cigs containing flavor and benzaldehyde could impair the phagocytic activity of neutrophils (Hickman *et al.* 2019).

Excessive ROS can also stimulate the continuity of activation in transcription factors such as nuclear factor erythroid two related factor 2 (Nrf2), hypoxia-inducible factor 1α (HIF-1α), activator protein 1 (AP-1), and nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) (Averill-Bates 2024), leading to stress-induced gene expression, cancer development, disrupting cellular homeostasis, and exaggerating inflammatory responses (Hong *et al.* 2024). Additionally, ROS accumulation can also decrease the activity of androgenic enzymes, such as 3β-hydroxysteroid dehydrogenase (3β-HSD) and 17β-hydroxysteroid dehydrogenase (17β-HSD). Those two steroid enzymes are essential for testosterone biosynthesis in the testes (Ullah *et al.* 2018), which is necessary for sperm maturation. Indeed, failure in the maturation of sperm development may lead to teratozoospermia, reflected in an increase in abnormal sperm morphology (Hussein 2018). Undoubtedly, our findings regarding the presence of micronucleated erythrocytes caused by DNA damage and increasing sperm abnormalities are in line with the molecular prediction test in this study, where genes related to oxidative stress were found to be increased.

In conclusion, based on our results, nicotine-free vapor is less harmful than nicotine-containing vapor for quitting tobacco smoking. However, long-term use is still questionable. Chronic exposure to nicotine-free vapor should be a future study. Biomarkers of disorder genes in abnormal sperm and in micronucleated cells, as well as reactive oxygen species markers in long-term exposure, should be identified to provide more comprehensive data.

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