Vanillin flavor in e-liquids does not induce endothelial cell dysfunction: the Replica Project.

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Keywords: flavor, vanillin, e-liquids, toxicity, endothelial dysfunction

Abstract

The e-cigarette seems to be a safer alternative to tobacco combustible cigarette and a valuable tool to reduce the tobacco harm caused by smoke. It is a battery-powered device that simulates smoking by heating an e-liquid to produce an aerosol that the user inhales. E-liquid typically contains a combination of propylene glycol (PG), vegetable glycerin (VG), nicotine, and chemical flavors. Flavors are generally recognized as safe (GRAS) for food applications, but little is known about their application in vaping. In this study the international Replica group investigated the effects of vanillin, one of the most widespread flavors in vaping, on the endothelium when vaporized from an e-cigarette. We vaped e-liquids containing PG, VG, and vanillin in two settings, regular and sub-ohm, to verify how vanillin behaves towards aortic endothelial cells, to replicate a study conducted by Fetterman and colleagues in 2018. We evaluated cytotoxicity, oxidative stress, and nitric oxide bioavailability, by covering some gaps reported in the original study by Fetterman. We observed a certain harmful effect mostly attributable to ethanol, mistakenly used to dilute vanillin in the original work by Fetterman and colleagues, but no harmful effect either on the viability of the cells or on their ability to produce nitric oxide. Even a certain protective effect against oxidative stress for vanillin has been observed. Our results confirm the endothelial cell dysfunction observed in the original paper but clarify that these observations are attributable to the ethanol and not to vanillin, which instead, in a more appropriate and realistic model of exposure, seems to exert a protective effect, particularly in a regular setting of the e-cigarette, compared to the most extreme setting (sub-ohm).

Introduction

The World Health Organization (WHO) estimates that 1.1 billion people smoke tobacco worldwide, with low- and middle-income countries accounting for about 80% of this population. The nations with the greatest smoking rates are usually those with less stringent tobacco control laws, like Indonesia, where almost 70% of men smoke (1).

Some nations have seen a decrease in smoking rates in recent years, partly as a result of the introduction of public awareness programs and tobacco control laws and partly for the introduction of alternative devices in the market (2, 3). Nonetheless, tobacco use continues to be a major global public health concern, and initiatives are being made to lower the prevalence of smoking and lessen the harmful consequences of tobacco use.

Efforts and tactics to lessen the harmful health impacts of smoking tobacco products, including cigarettes, are referred to as tobacco harm reduction (THR) (4). While quitting smoking is the most effective strategy to reduce the dangers connected with it, harm reduction approaches recognize that some people may be unable or unwilling to quit and strive to mitigate the harm caused by smoking. Therefore, the purpose of THR is to minimize exposure to the dangerous chemicals and contaminants contained in tobacco smoke, which can lead to a variety of major health concerns including cancer, heart disease, and respiratory disorders. The WHO acknowledges that some alternative nicotine products, such as e-cigarettes, have the potential to reduce the harm caused by tobacco use, despite concerns about the long-term health effects of these products, particularly among young people.

The e-cigarette is a battery-powered device that simulates smoking by heating a liquid solution (commonly known as e-liquid or vape juice) to produce an aerosol that the user inhales. E-liquid typically contains a combination of propylene glycol, vegetable glycerin, nicotine, and flavoring.

The observation that e-cigarette aerosol shows a significant decrease by more than 80% (5, 6) in cytotoxicity compared to tobacco smoke suggests that e-cigarettes may be a less harmful alternative to traditional cigarette smoking, at least in terms of potential impact on cell health.

In addition to the promising finding of reduced cytotoxicity, numerous clinical studies show similar data on smokers who switch to e-cigarettes (7-9). However, more research is needed to fully understand the health effects of e-cigarettes and the potential risks associated with their prolonged use. E-liquids are available in a wide variety of flavors, from traditional tobacco and menthol to fruit, desserts, candies, beverages, and even unconventional flavors such as cereal or pastry, to suit different preferences (10, 11). These flavors are an essential component of the vaping experience and are created using a combination of food-grade flavors, PG and VG. Moreover, e-liquids are available in various nicotine strengths, including nicotine-free options. User preferences for flavors can vary

greatly, and finding the right flavor is a subjective and personal experience. Regulation of vaping products, including e-liquids, varies by region.

The safety of flavors in vaping largely depends on the quality of the ingredients and recognized e-liquid manufacturers use food flavorings generally recognized as safe (GRAS) (12). These flavors are tested for safety in food products, but it is important to note that inhaling these substances into the lungs can have different effects than ingesting them. Some concerns have been raised about specific compounds used in flavorings, such as diacetyl, acetyl propionyl and acetoin, which may be associated with respiratory problems if inhaled (12). Many of the most used flavors in e-liquids have been studied with various approaches with the purpose of determine their safety in vaping and tobacco products (13-16). In one of these studies, some flavors, including vanillin, showed potential to induce endothelial cell dysfunction when vaporized. Unfortunately, many of these studies do not provide strong evidence of the use of these flavors in electronic cigarettes because they do not faithfully reconstruct the vaporization process that occurs in e-cigarettes. For this reason, we decided to replicate one of these studies, Fetterman et al. 2018 (15), by testing one of the most widespread flavors, such as vanillin, and using standardized methods, a regular vaping machine and real e-cigarettes to verify whether the results obtained were applicable to the real vaping procedure. As is habitual with the Replica study, the experiments were performed independently in four laboratories in the world, following the standardized and harmonized SOPs between the project partners (5, 6, 17).

Materials and methods

Study design and Harmonization process

This is an interlaboratory *in vitro* study conducted in the framework of the new phase of REPLICA 2.0 project. The multi-center research network established for this study includes laboratories from Italy (LAB-A) – the leading center –, USA (LAB-B), Indonesia (LAB-C), and Serbia (LAB-D).

As recommended by the Centre for Open Science transparency and openness promotion guidelines (https://www.cos.io/initiatives/top-guidelines), protocols were standardized across laboratories with standard operative procedures (SOPs) defined for each experimental step. Moreover, all laboratories used the same cell line, cell-exposure equipment, and methods to assess endpoints. LAB-A arranged a kick-off meeting to train the staff of international partners and harmonize the SOPs. These SOPs have been designed to be as close as possible to the protocols of the original paper, except for the vanillin vaporization and cytotoxicity assay. Particularly, the major limitations stated by the authors of Fetterman et al.(15) were the use of flavor diluted in ethanol and not in propylene glycol (PG) and

glycerol (VG), and their heating without using an electronic cigarette and a standardized exposure system. We covered these gaps by dissolving the vanillin in PG and VG solvents and using an electronic cigarette vaped by a standardized vaping machine (LM4E; Borgwaldt; Hamburg, Germany). Another difference from the original paper is the use of neutral red uptake assay and MTS assay instead of TUNEL for the evaluation of cytotoxicity.

Cell culture

The experiments were performed using the Human Aortic Endothelial Cells (HAEC) purchased from Lonza (CC-2535). Cells were cultured in EBM™-2 Basal Medium (Lonza, CC-3156) supplemented with EGM™-2 SingleQuots™ (Lonza, CC-4176), and incubated in a humidified atmosphere (5% CO_2) at 37 °C. All experiments with HAECs were performed within passages 2 - 4. When the cells reached confluence, they were detached with trypsin–EDTA solution (0.05%) and replated in new flasks (cell passaging), or into 96-well plates (cell viability assay, Nitric Oxide bioavailability, oxidative stress assessment), or into 12-well plates (RT-qPCR). The cells were received by the vendor in a frozen vial, and the thawed cells have been considered as "passage 0".

Test products and preparation of e-liquids

The e-cigs used were Aspire Zelos 3 bought from Italian dealers. For the preparation of the e-liquids were used propylene glycol (PG) (Sigma Aldrich, P4347-500ML), vegetable glycerol (VG) (Sigma Aldrich, 1370282500), vanillin (Sigma Aldrich, V1104-100G), and absolute ethanol (Sigma Aldrich, 1009832500). The day before the exposure run were prepared 4 different liquids: PG/VG 50:50 (50% of PG and 50% of VG), PG/VG 50:50 with Vanillin (50% of PG and 50% of VG at 200 mM of vanillin), PG/VG 30:70 (30% of PG and 70% of VG), and PG/VG 30:70 with Vanillin (30% of PG and 70% of VG at 200 mM of vanillin). Vanillin was first diluted with absolute ethanol to a concentration of 2 M and then was mixed with PG and VG. PG/VG 50:50 and PG/VG 50:50 with Vanillin were used with e-cig set in Wattage control mode (1.0 Ohm coil and 14 Watt) and the MTL (mouth-to-lung) drip tip inserted. PG/VG 30:70 and PG/VG 30:70 with Vanillin were used with e-cig set in Temperature control mode (0.3 Ohm coil and 200 °C) and the DTL (direct-to-lung) drip tip inserted.

Vapor exposure

The different e-liquids loaded to the e-cigs were vaped by the LM4E Vaping Machine (Borgwaldt, Hamburg, Germany) following the "CORESTA Reference Method n. 81" (CRM81) regimen (55 ml puff volume, drawn over 3 s, once every 30 s with square shaped profile, and with a puff velocity of

18.3 ml/s), accredited into ISO 20768:2018 (18). A button pre-activation of 1 s was also applied as per guidelines CRM81. The standard exhaust time for LM4E was 0.7 s with a flow rate in the impinger of 78.57 ml/s. The vapor was bubbled into an impinger filled with 30 ml of trapping solution (20% of absolute ethanol and 80% of PBS) to prepare the aqueous extracts (AqEs) for treatments. The laboratory conditions were checked using temperature and humidity sensors prior and during the exposure, maintaining a relative humidity of 40-70% and a temperature between 15 °C and 25 °C ±2 °C, according to ISO 20768 (18).

Neutral Red Uptake (NRU) assay

24 hours prior to AqEs treatment, HAECs were trypsinized (0.05% Trypsin/0.02% EDTA), resuspended in EGM™-2 complete medium, seeded in a 96-well plate at a density of 10 x 10^3 viable cells/well, and incubated at 37 °C (5% CO2, humidified atmosphere). After vapor exposure, AqEs were filtered with 0.2 µm syringe filter. Then the cells were treated with seven different dilutions (1:2 or 100 mM, 1:4 or 50 mM, 1:8 or 25 mM, 1:20 or 10 mM, 1:200 or 1 mM, 1:2000 or 0.1 mM, 1:20000 or 0.01 mM) of PG/VG 50:50 AqE, PG/VG 50:50 with Vanillin AqE, PG/VG 30:70 AqE, and PG/VG 30:70 with Vanillin AqE for 24 hours. Following Fetterman's indications (15), the molarity concentration reported and the relative dilutions of the AqE are referred to the initial concentration of Vanillin (200 mM) calculated in the relative liquids. For the dilution of AqEs was used EGM™-2 complete medium with 20 mM HEPES (Gibco, 15630-080). Next, the cells were washed with PBS and incubated with Neutral Red Solution (0.05 g/L; Sigma, N2889-100ML) diluted in EGM™-2 complete medium with 20 mM HEPES at 37 °C (5% CO2, humidified atmosphere) for 3 hours. Afterwards, the cells were washed with PBS, and the Neutral Red dye retained by cells was extracted by the addition of destaining solution (ethanol, distilled water, and acetic acid at a ratio of 50:49:1, respectively). Subsequently, the plates were shaken using an orbital agitator for 10 min at 300 rpm. Eventually, Neutral Red absorbance was measured in a microplate reader at 540 nm using a reference filter of 630 nm.

MTS assay

Cell seeding and treatment procedures were performed as previously stated for the NRU assay. After 24 hours of incubation with AqEs, the treatment was removed from the wells and the cells were incubated with 100 µl of EGM™-2 complete medium with 20 mM HEPES and 20 µl of MTS (Promega, CellTiter 96® AQueous One Solution Reagent) at 37 °C (5% CO2, humidified atmosphere) for 3 hours. The absorbance of soluble formazan produced by cellular reduction of MTS was measured using a microplate reader at 490 nm.

Nitric Oxide Bioavailability

HAECs were seeded in a 96-well plate at a density of 10 x 10^3 cells/well and incubated at 37°C (5% CO2, humidified atmosphere) for 24 hours, until 80-90% confluence. Then the cells were exposed for 90 minutes to four different dilutions (1:20 or 10 mM, 1:200 or 1 mM, 1:2000 or 0.1 mM, and 1:20000 or 0.01 mM) of PG/VG 50:50 AqE, PG/VG 50:50 with Vanillin AqE, PG/VG 30:70 AqE, and PG/VG 30:70 with Vanillin AqE. The reported molarity concentration and dilutions of AqEs were established basing on the initial Vanillin concentration of the related products (200 mM). Prior to dilution, AqEs were filtered with 0.2 µm syringe filter. For the dilution was used EGM™-2 complete medium with 20 mM HEPES. Next, the cells were incubated for 30 minutes with 3 µmol/L 4,5-diaminofluorescein diacetate (DAF-2 DA; Sigma-Aldrich, D225). After that, the cells were washed 2 times with pre-warmed DPBS, stimulated for 15 minutes with 1 µmol/L Calcium Ionophore A23187 (Sigma-Aldrich, C7522), and fixed with 2% paraformaldehyde in DPBS for 10 minutes at room temperature. Afterwards, paraformaldehyde was replaced with 200 µl of DPBS to each well and the fluorescence intensity (excitation of 492 nm, emission peak at 515 nm) was measured by a microplate reader. Data are presented as percentage increase in DAF-2 DA fluorescence stimulated by Calcium Ionophore A23187 compared with unstimulated cells.

Oxidative Stress assessment

Cell seeding and exposure to AqE solutions were performed as explained above (Nitric Oxide bioavailability). Next, HAECs were incubated for 30 minutes with 10 µmol/L dihydroethidium (DHE; Thermo Fisher Scientific, D11347). After that, HAECs were washed 3 times with pre-warmed DPBS to remove DHE. As a positive control was used 50 µmol/L Antimycin A (Sigma-Aldrich, A8674) for 30 minutes. The fluorescence intensity was measured within 30 minutes using a microplate reader with excitation of 518 nm and emission of 606 nm. Data are shown as fold change in DHE fluorescence in comparison with vehicle control.

Statistics

Microsoft Excel was used to tabulate and process all of the raw data. The Shapiro-Wilk test was used for assessing the normality or skewness of data distribution. The outlier detection was made using the confidence interval at 95% of the median or the robust regression-based outlier rejection (ROUT) test. All data were reported as median (Interquartile range – IQR). The Kruskal-Wallis test was applied to determine any statistically significant differences between the medians of study groups. Additionally, post-hoc Dunn's test was performed for a more detailed examination of group

differences after obtaining significant Kruskal-Wallis results. All analyses were considered significant with a p value of less than 5 %. GraphPad Prism 8 software was used for data analysis and generation of graphs unless otherwise stated.

Results

Cytotoxicity evaluation of Vanillin

Cytotoxicity of HAECs exposed to PG/VG Vanillin for both regular and sub-ohm settings was evaluated by NRU and MTS assays after 24 hours of treatments, in contrast to Fetterman's assessment at 90 minutes. The evaluation of cytotoxicity induced with regular setting by NRU showed significant decrease of HAECs viability after exposure to 100 mM of Vehicle control (p= 0.005), PG/VG base (p= 0.007) and PG/VG Vanillin (p= 0.005) compared to control medium. No significant differences were observed between each concentration of both PG/VG base and PG/VG Vanillin and the corresponding vehicle control concentration (Fig 1-A). NRU data normalized to vehicle control (Fig 1-B) revealed no significant differences among test products and vehicle controls. Moreover, no significant differences in cell viability were observed among PV/VG base and PG/VG Vanillin compared at each concentration (Fig 1-B).

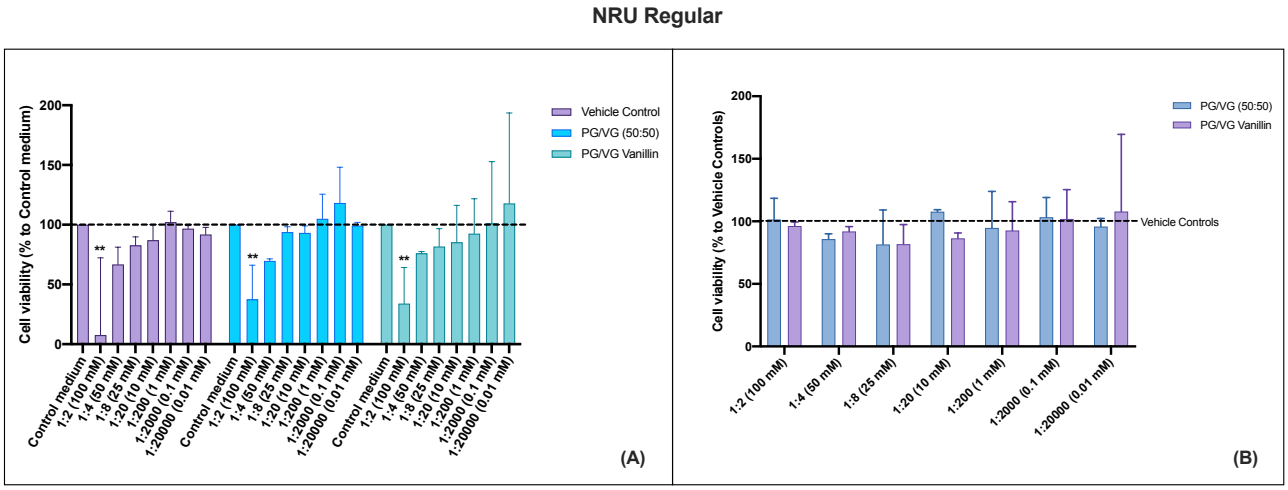

Figure 1. HAECs cytotoxicity evaluation by NRU assay of PG/VG base and PG/VG Vanillin using the regular setting at 24 hours. (**A**) NRU data normalized as percentage of control medium. The dashed line corresponds to control medium. (**B**) NRU data normalized as percentage of corresponding Vehicle control concentration. The dashed line corresponds to Vehicle control. All data are reported as median (IQR). ** p< 0.01.

The evaluation of cytotoxicity induced with sub-ohm setting assessed by NRU assay demonstrated significant decreases of HAECs viability not only after exposure to 100 mM of Vehicle control (p< 0.0001), PG/VG base (p< 0.0001) and PG/VG Vanillin (p< 0.0001) but also to 50 mM of Vehicle

control (p= 0.0008), PG/VG base (p= 0.0009) and PG/VG Vanillin (p= 0.0003), 25 mM of PG/VG base (p= 0.019) and PG/VG Vanillin (p= 0.012), and 10 mM of PG/VG Vanillin (p= 0.0395) compared to control medium. (Fig 2-A). The NRU data normalization to vehicle control (Fig 1-B) indicated no significant differences among test products and vehicle controls. Moreover, the comparison between PV/VG base and PG/VG Vanillin at each concentration illustrated a significant difference between PV/VG base and PG/VG Vanillin at 100 mM. No significant differences in cell viability were observed for the other comparisons (Fig 1-B).

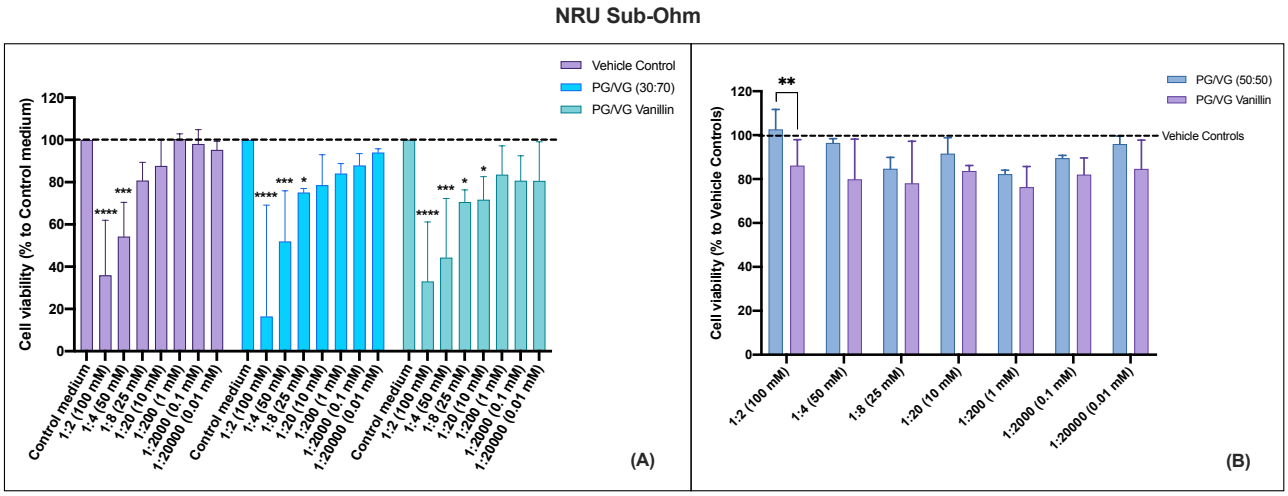

Figure 2. HAECs cytotoxicity evaluation by NRU assay of PG/VG base and PG/VG Vanillin using the sub-ohm setting at 24 hours. (**A**) NRU data normalized as percentage of control medium. The dashed line corresponds to control medium. (**B**) NRU data normalized as percentage of corresponding Vehicle control concentration. The dashed line corresponds to Vehicle control. All data are reported as median (IQR). *p< 0.05; ** p< 0.01; ***p< 0.001; ****p< 0.0001.

Similar results were observed when the MTS assay was used for the cytotoxicity evaluation in HAECs exposed to PG/VG and PG/VG Vanillin. The MTS assay was carried out only by LAB-A and LAB-B, thus having two independent replicants of each experiment. The assessment of cytotoxicity induced under regular setting, as determined by the MTS assay, demonstrated a reduction in the viability of HAECs following exposure to 100 mM concentrations of Vehicle control (p< 0.0001), PG/VG base (p< 0.0001), and PG/VG Vanillin (p< 0.0001) in comparison to the control medium. The 50 mM concentration of Vehicle control (p= 0.015), PG/VG base (p= 0.016), and PG/VG Vanillin (p p= 0.044) induced also a slight decrease compared to control medium. No significant variations were noted between each concentration of both PG/VG base and PG/VG Vanillin and their respective vehicle control concentrations (Fig 3-A). Normalizing the MTS data to the vehicle control (Fig 3-B) revealed no significant differences among the test products and their corresponding vehicle controls. Additionally, no notable differences in cell viability were observed when comparing PV/VG base and PG/VG Vanillin at each concentration (Fig 3-B).

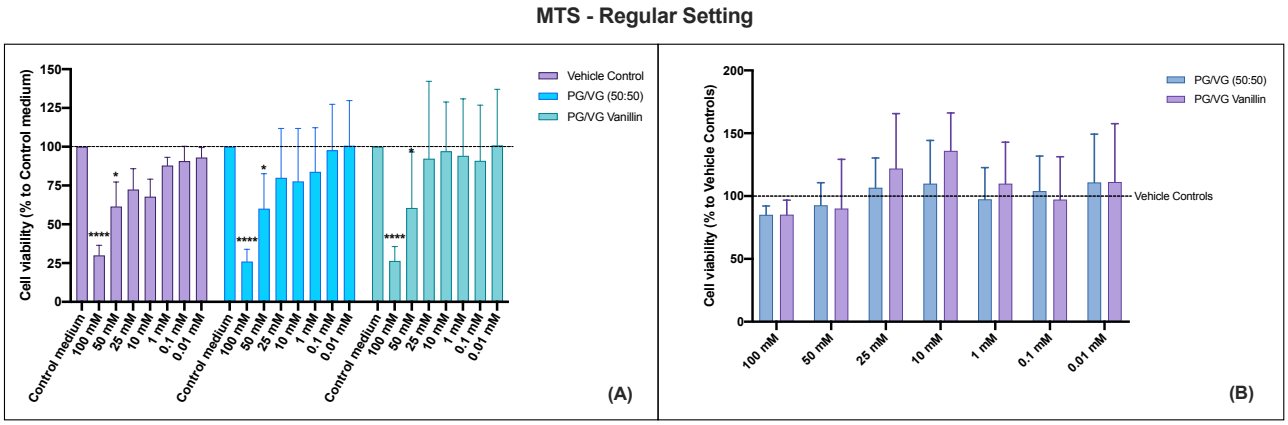

Figure 3. HAECs cytotoxicity evaluation by MTS assay of PG/VG base and PG/VG Vanillin using the regular setting at 24 hours. (**A**) MTS data normalized as percentage of control medium. The dashed line corresponds to control medium. (**B**) MTS data normalized as percentage of corresponding Vehicle control concentration. The dashed line corresponds to Vehicle control. All data are reported as median (IQR). *p< 0.05; **** p< 0.0001.

The assessment of cytotoxicity induced through sub-ohm settings by the MTS assay revealed more significant reductions in the viability of HAECs. The decrease was evident not only after exposure to 100 mM concentrations of Vehicle control (p< 0.0001), PG/VG base (p< 0.0001), and PG/VG Vanillin (p< 0.0001) but also with 50 mM concentrations of Vehicle control (p< 0.0001), PG/VG base (p= 0.0001), and PG/VG Vanillin (p< 0.0001), 25 mM of Vehicle control (p= 0.0007) PG/VG base (p= 0.002) and PG/VG Vanillin (p= 0.003), and 10 mM of Vehicle control (p= 0.014) compared to the control medium (Fig 2-A). Normalizing the MTS data to the vehicle control (Fig 1-B) revealed no significant differences among the test products and their respective vehicle controls. Furthermore, the comparison between PG/VG base and PG/VG Vanillin at each concentration indicated no significant variations in cell viability (Fig 1-B).

All these results demonstrate that the cytotoxic effect on HAECs is predominantly due to the ethanol present in the vehicle control. In fact, normalization of the data to the respective ethanol concentrations reveals no cytotoxic effect of the aerosols with and without vanillin.

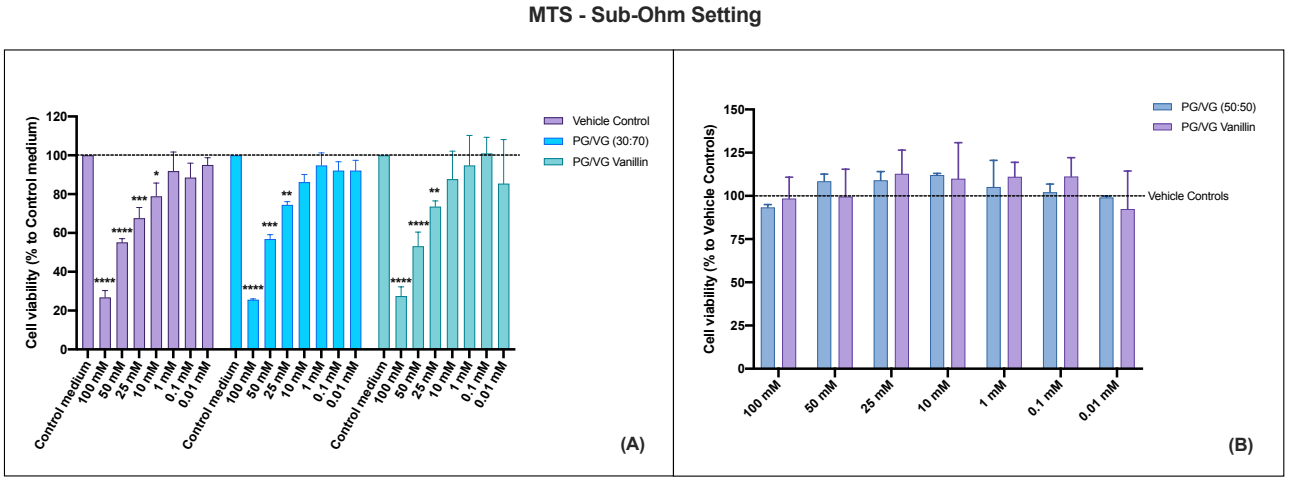

Figure 4. HAECs cytotoxicity evaluation by MTS assay of PG/VG base and PG/VG Vanillin using the sub-ohm setting at 24 hours. (**A**) MTS data normalized as percentage of control medium. The dashed line corresponds to control medium. (**B**) MTS data normalized as percentage of corresponding Vehicle control concentration. The dashed line corresponds to Vehicle control. All data are reported as median (IQR). *p< 0.05; ** p< 0.01; ***p< 0.001; ****p< 0.0001.

Oxidative Stress

Following 90 minutes HAECs' treatment with PG/VG and PG/VG Vanillin at various doses, oxidative damage was measured using the fluorescent dye DHE, as reported by Fetterman and colleagues (15). The positive control, Antimycin A, raised the amounts of oxidants as indicated by an increase in DHE fluorescence compared to control medium in the evaluation of both settings, regular (p= 0.045) and sub-ohm (p= 0.009). The assessment of oxidative stress for the regular setting revealed no significant difference between the different conditions tested in comparison to the medium control (Fig. 5A). While there seems to be an increase in oxidative stress response following exposure to the vehicle control and unflavored PG/VG, the considerable variability observed in the results likely precludes the statistical demonstration. Instead, assessing the sub-ohm setting, we observed significant rise in oxidative stress after 90 minutes treatment with 10 mM of Vehicle control (p= 0.024) and PG/VG (p= 0.015), and 1 mM of Vehicle control (p= 0.031) and PG/VG (p= 0.025) compared to control medium (Fig. 6A). The treatment with PG/VG Vanillin did not reveal significant increase in oxidative stress compared to control medium. Moreover, the data normalization to the corresponding Vehicle control concentrations (Fig. 5B and 6B) indicated no significant differences between unflavored PG/VG and PG/VG Vanillin compared to vehicle controls and even in comparison to each other.

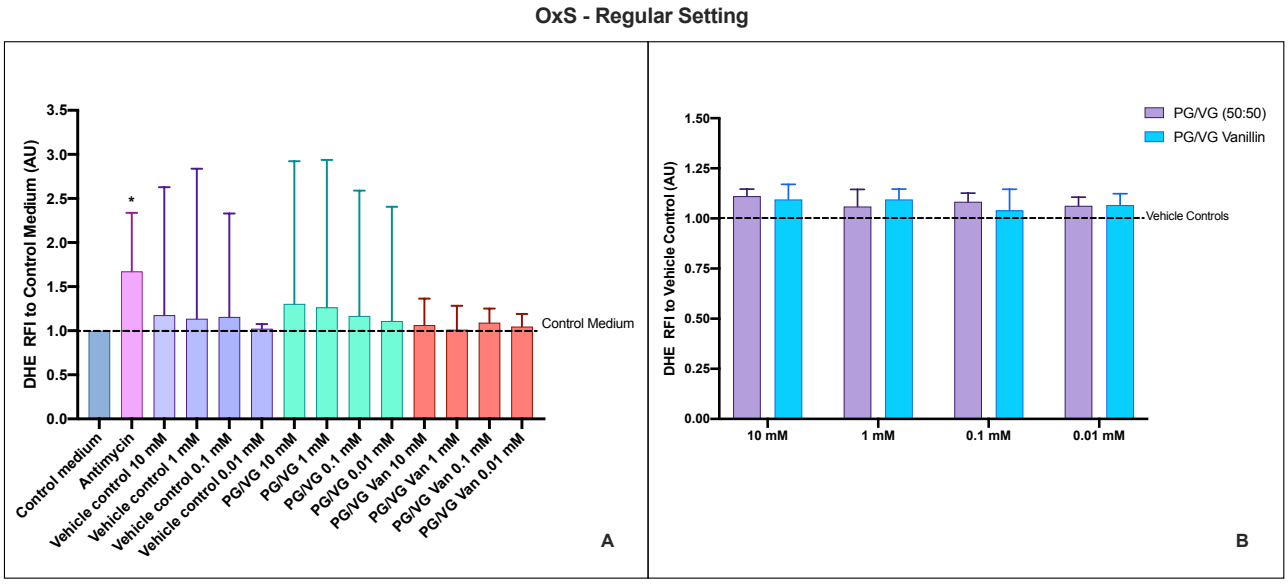

Figure 5. HAECs Oxidative Stress (OxS) evaluation by DHE of PG/VG base and PG/VG Vanillin using the regular setting. (**A**) OxS data normalized to control medium. The dashed line corresponds to control medium. (**B**) OxS data normalized to the corresponding Vehicle control concentration. The dashed line corresponds to Vehicle control. All data are reported as median (IQR). *p< 0.05.

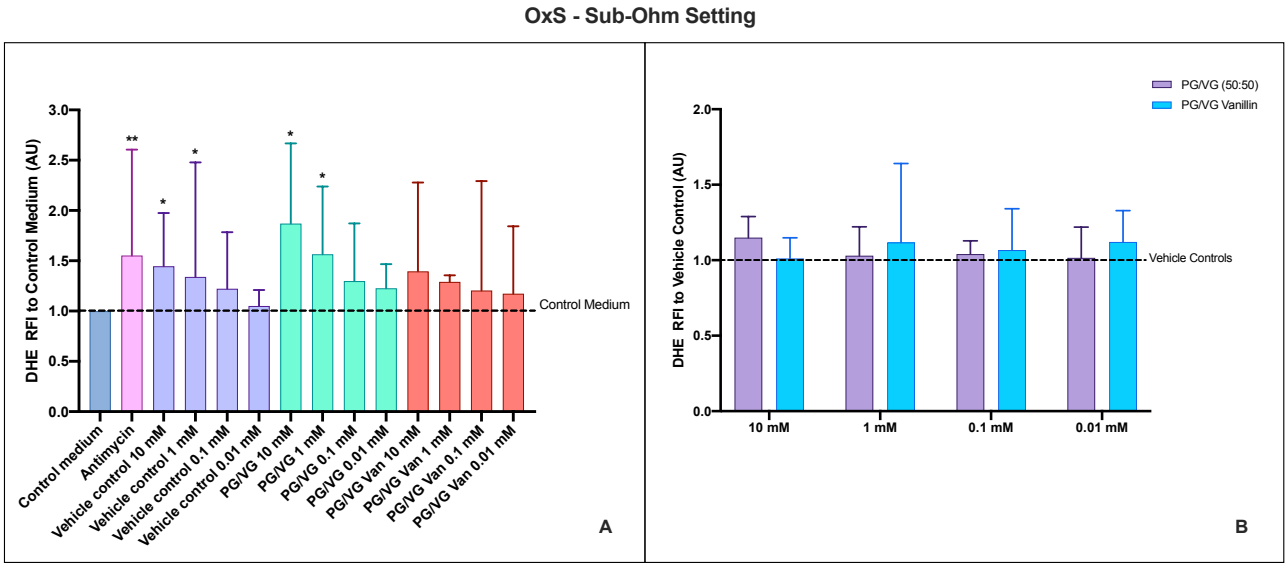

Figure 6. HAECs Oxidative Stress (OxS) evaluation by DHE of PG/VG base and PG/VG Vanillin using the regular setting. (**A**) OxS data normalized to control medium. The dashed line corresponds to control medium. (**B**) OxS data normalized to the corresponding Vehicle control concentration. The dashed line corresponds to Vehicle control. All data are reported as median (IQR). ** p< 0.01; *p< 0.05.

<u>Nitric Oxide Bioavailability was not impaired by Vanillin</u>

As carried out by Fetterman and colleagues (15), we evaluated the effect of vanillin on nitric oxide production by HAECs in response to A23187 stimulation. No significant differences were observed between PG/VG and PG/VG Vanillin and the respective Vehicle Control concentrations for both regular (Fig. 7) and sub-ohm settings (Fig. 8). In some cases, it appears that only PG/VG exposure

may exert a minor impact on NO production, though this is not significant compared to vehicle control. Instead, the exposure to PG/VG Vanillin appears to improve the bioavailability of NO compared to PG/VG, although no significant differences were observed among PG/VG and PG/VG Vanillin.

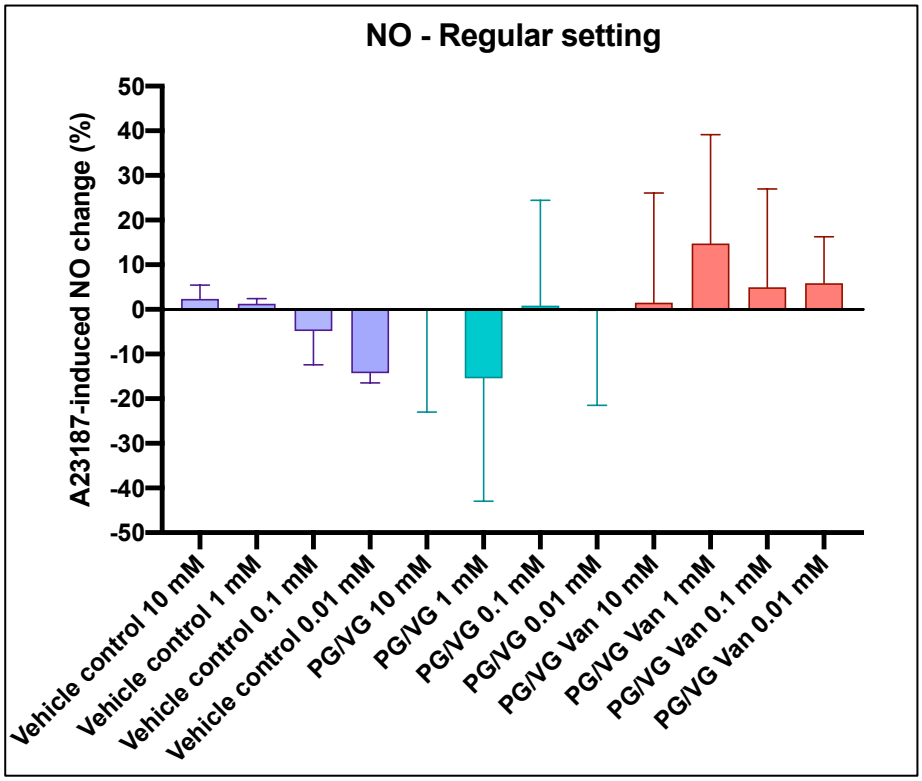

Figure 7. Effects of PG/VG and PG/VG Vanillin on A23187-stimulated nitric oxide production in human aortic endothelial cells (HAECs) using Regular Setting. All data are normalized as percentage of A23187-induced NO change and reported as median (IQR).

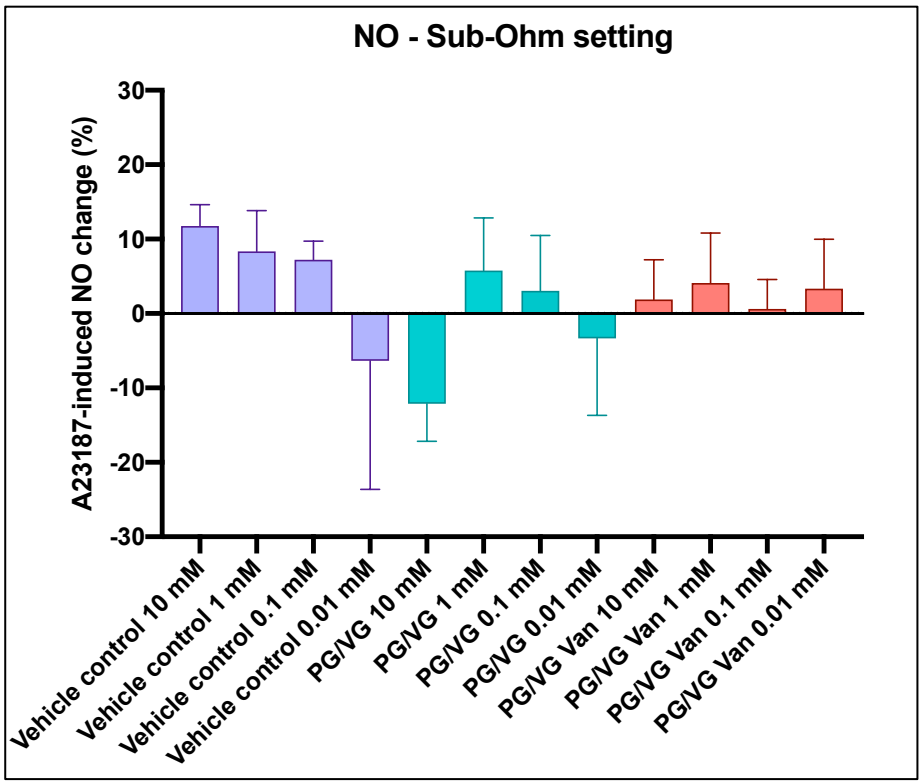

Figure 8. Effects of PG/VG and PG/VG Vanillin on A23187-stimulated nitric oxide production in human aortic endothelial cells (HAECs) using Sub-Ohm Setting. All data are normalized as percentage of A23187-induced NO change and reported as median (IQR).

Discussion

In our attempt to replicate the experiments conducted by Fetterman et al., 2018 (15) in their paper titled "Flavorings in Tobacco Products Induce Endothelial Cell Dysfunction," our focus was on investigating the impact of vanillin used in e-liquids on endothelial cell function, evaluating the cytotoxicity, oxidative stress, and bioavailability of nitric oxide. While the original study provided evidence that vanillin flavoring induces acute alterations in endothelial functions, our replication efforts revealed different results and covered some methodological gaps mainly concerning the method of exposure.

One of the key aspects in our replication was the meticulous alignment of our methods with those outlined in the original study and specified personally by the corresponding author. However, despite our efforts to adhere closely to their experimental procedures, we identified several methodological discrepancies that could have contributed to the disparities in our results. Notably, the use of TUNEL assay that is not typically used for the direct evaluation of cytotoxicity, but it is a method commonly employed for detecting apoptotic cell death (19). Then, TUNEL assay itself does not provide a comprehensive assessment of general cytotoxic effects. For this reason, we performed NRU and MTS assays, which provide information about overall cell damage, regardless of the specific mechanism of cell death (20). Moreover, we covered the main limitation mentioned by the authors, which is the

flavor dilution in ethanol rather than propylene glycol (PG) or glycerol (VG), as well as heating them without the use of an electronic cigarette and a standardized exposure mechanism. Indeed, we prepared e-liquids by dissolving the vanillin in PG and VG solvents and heating the e-liquid through an electronic cigarette that was vaped using a standardized vaping machine. We maintained the composition of trapping solution (Ethanol/PBS – 20:80%), as indicated by the corresponding author. Our results from both NRU and MTS revealed a cytotoxic effect related to the ethanol present in the vehicle control rather than the presence of vanillin in the aqueous extract. It is recognized that ethanol has a cytotoxic effect on cells, especially true at higher concentrations (21). This is why we included a vehicle control for each concentration used in the assays, demonstrating that the apparent cytotoxicity of PG/VG and PG/VG with vanillin was mainly due to the presence of ethanol. Beyond Fetterman's work, there are no other data in the literature indicating cytotoxicity of vanillin, except for cancer cells (22). In fact, vanillin has been extensively studied as an anti-cancer agent mainly for its anti-proliferative and protective properties against DNA-damaging agents (23, 24).

Another important property of vanillin is its antioxidant and anti-inflammatory activity (23, 25). Vanillin is reported as a potent scavenger of reactive oxygen species (ROS) and an inhibitor of inducible nitric oxide synthase (iNOS) (26). Assessing oxidative stress, Fetterman and colleagues did not observe a significant increase in ROS after treatment with vanillin. Likewise, our results indicated that vanillin has no pro-oxidant effect on aortic endothelial cells. Furthermore, it is essential to note that our investigation revealed an antioxidant effect, albeit not a very pronounced one, following exposure to vanillin especially in the regular setting. Indeed, vanillin-treated cells did not show a significant increase in ROS levels, indicative of its antioxidant influence. In contrast to the vehicle control, in which a discrete increase in ROS levels was noted, PG/VG vanillin-treated cells did not reveal an increase in oxidative stress beyond baseline levels.

The bioavailability of nitric oxide (NO) is a critical determinant of endothelial function and play a central role in the maintenance of vascular homeostasis with its strong vasodilatory, anti-inflammatory, and antioxidant properties (27). Decrease in NO levels have been implicated in endothelial dysfunction, leading to prothrombotic, proinflammatory, and less compliant blood vessel wall (27, 28). Our investigation into the bioavailability of NO in aortic endothelial cells yielded intriguing results, as we found no significant changes in NO levels in endothelial cells treated with PG/VG vanillin. This contrasts with the reported decrease in NO bioavailability by Fetterman and colleagues, which reported that vanillin impaired NO production of aortic endothelial cells in response to A23187 stimulation, highlighting discrepancies with existing literature data. Vanillin is well known as a cardioprotective substance (24). Indeed, it has been found to stimulate dose-dependent relaxation of isometric tensions during coronary artery contractions induced by different

muscle contraction-inducing agents (29). Furthermore, oral administration of vanillin in mice revealed a cardioprotective effect in that it reduced cardiac protein oxidation and lipid peroxidation and improved cardiac morphology (30). Heating the vanillin contained in e-liquids could lead to the formation of substances that could impair the endothelial NO balance state but, as our results show, acute exposure to PG/VG vanillin under close-to-realistic conditions resulted in no NO reduction in aortic endothelial cells. Finally, while commonly used in various scientific applications, ethanol poses potential challenges that require careful consideration, especially when conducting *in vitro* experiments. Cytotoxic and pro-oxidant effects, and potential interference with analytical systems are important aspects to consider when evaluating results. It is also essential to consider the ethanol evaporation that researchers must deal with. As also observed during the execution of our experiments, ethanol evaporation brought negative implications for the reliability and reproducibility of results with high intra- and inter-laboratory variability.

In conclusion, our replication attempt of the experiments conducted by Fetterman et al., 2018 (15), which focused on the impact of vanillin in e-liquids on endothelial cell function, revealed significant disparities in the results compared to the original study. Despite our meticulous efforts to align with the specified experimental procedures, methodological discrepancies, particularly the use of a standardized exposure method closer to the real vaping conditions and the choice to test the vehicle control at different concentrations, emerged as critical factors contributing to the observed variations. Further research is needed to refine our understanding of the potential health effects associated with flavorings in e-liquids. Careful consideration of methodological aspects, particularly the choice of assays and exposure method, is critical for reliable interpretation of results in studies of this nature.

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Funding

This investigator-initiated study was sponsored by ECLAT srl, a spin-off of the University of Catania, through a grant from the Foundation for a Smoke-Free World Inc., a US nonprofit 501(c)(3) private foundation with a mission to end smoking in this generation. The contents, selection, and presentation of facts, as well as any opinions expressed herein are the sole responsibility of the authors and under no circumstances shall be regarded as reflecting the positions of the Foundation for a Smoke-Free World, Inc. ECLAT srl. is a research-based company that delivers solutions to global health problems with special emphasis on harm minimization and technological innovation.

Conflict of interest statement

Riccardo Polosa is full tenured professor of Internal Medicine at the University of Catania (Italy) and Medical Director of the Institute for Internal Medicine and Clinical Immunology at the same University. He has received grants from U-BIOPRED and AIR-PROM, Integral Rheumatology & Immunology Specialists Network (IRIS), Foundation for a Smoke Free World, Pfizer, GlaxoSmithKline, CV Therapeutics, NeuroSearch A/S, Sandoz, Merk Sharp & Dohme, Boehringer Ingelheim, Novartis, Arbi Group Srl., Duska Therapeutics, Forest Laboratories, Ministero dell Universita' e della Ricerca (MUR) Bando PNRR 3277/2021 (CUP E63C22000900006) and 341/2022 (CUP E63C22002080006), funded by NextGenerationEU of the European Union (EU), and the ministerial grant PON REACT-EU 2021 GREEN- Bando 3411/2021 by Ministero dell Universita' e (MUR) – PNRR EU Community. He is founder of the Center for Tobacco Prevention and Treatment (CPCT) at the University of Catania and of the Center of Excellence for the Acceleration of Harm Reduction at the same university. He receives consultancy fees from Pfizer, Boehringer Ingelheim, Duska Therapeutics, Forest Laboratories, CV Therapeutics, Sermo Inc., GRG Health, Clarivate Analytics, Guidepoint Expert Network, and GLG Group. He receives textbooks royalties from Elsevier. He is also involved in a patent application for ECLAT Srl. He is a pro bono scientific advisor for Lega Italiana Anti Fumo (LIAF) and the International Network of Nicotine Consumers Organizations (INNCO); and he is Chair of the European Technical Committee for Standardization on "Requirements and test methods for emissions of electronic cigarettes" (CEN/TC 437; WG4). Giovanni Li Volti is currently elected Director of the Center of Excellence for the acceleration of HArm Reduction (CoEHAR). The other authors have no relevant financial interests to disclose.