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In vitro cytoxicity profile of e-cigarette liquid samples on primary human bronchial epithelial cells

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Abstract

Cigarette smoke is associated to severe chronic diseases. The most harmful components of cigarette smoke derive from the combustion process, which are significantly reduced in the electronic cigarette aerosol, thus providing a valid option in harm reduction strategies. To develop safer products, it is therefore necessary to screen electronic cigarette liquids (e-liquids) to meet high safety standards defined by government regulations. The aim of the present study was to evaluate the presence of metal- and plastic-derived contaminants in four different commercial e-liquids with high concentration of nicotine and their cytotoxic effect in normal human bronchial epithelial cells by a number of in vitro assays, in comparison with the 1R6F reference cigarette, using an air-liquid interface (ALI) exposure system. Moreover, we evaluated the effect of aerosol exposure on oxidative stress by measuring the production of reactive oxygen species and mitochondrial potential. Our results showed no contaminants in all e-liquids and a significantly reduced cytotoxic effect of e-liquid aerosol compared to cigarette smoke as well as a maintained mitochondria integrity. Moreover, no production of reactive oxygen species was detected with e-cigarette aerosol. In conclusion, these results support the reduced toxicity potential of e-cigs compared to tobacco cigarettes in an in vitro model resembling real life smoke exposure.

KEYWORDS
aerosol, cytotoxicity, metals, microplastics, smoke

Abbreviations: CRM81, CORESTA Recommended Method n. 81; E-cigs, electronic cigarettes; e-cigs, electronic cigarettes; HCI, Health Canada Intensive; HCS, high content screening; ISO, International Organization for Standardization; RTCA, real time cell analysis; THPs, tobacco heating products.

Massimo Caruso and Alfio Distefano contributed equally to this work.

1 | INTRODUCTION

Cigarette smoke is associated to a number of diseases, most of which are represented by long-term effects on the respiratory and cardiovascular apparatus. These effects depend in part by the reiteration of acute insults to cells over the time, such as the epithelium of the respiratory system leading to chronic damage, inflammation, and tissue remodeling.

More than 7 million people die each year using combustible tobacco products, making smoking the leading cause of avoidable deaths worldwide.[1] Interventions aimed at reducing tobacco consumption have led to poor results, reducing the prevalence of tobacco users from 22.5% in 2007 to 19.2% in 2017 worldwide, with a very poor response in middle- and low-income countries.[1] In the last decade, the use of low-risk combustion-free alternatives, such as electronic cigarettes[2] (e-cigs), has grown worldwide. E-cigs include a wide range of various devices, consisting of a battery serving as an energy source and heating an atomizer wet by a liquid (e-liquid) which is then vaporized and thus delivering nicotine through the inhalation of an aerosol. Given that the most harmful components of cigarette smoke are produced by the combustion process, it is reasonable to assume that the use of e-cigs in place of tobacco cigarettes can reduce the burden of smoking-related morbidity and mortality by substantially reducing the chronic exposure to tar from cigarette smoke. The potential benefits and risks of using e-cig have been the matter of intense scientific debate.[3,4] In addition to the variety of devices on the market, an issue to be taken into due account regards the formulation of the liquids used to generate the aerosol and their toxicological effects. The e-liquid consists largely of propylene glycol (PG) and vegetable glycerin (VG), and a smaller part of food chemical flavorings and nicotine (which may also be absent).[5] The e-liquids are produced through the use of industrial processes and automated mechanical systems with metal and plastic parts and marketed in plastic containers, typically darkened to protect the nicotine which is photosensitive. This could lead to the presence of heavy metal residues, such as arsenic, lead, aluminum, iron, mercury and cadmium, or nanoplastics (NPs) and microplastics (MPs) in the liquids themselves. Heavy metals contamination of e-liquids could be responsible to produce oxidative species and subsequent adverse effects on health of consumers.[6] Moreover, tissue accumulation of heavy metals leads to an imbalance and can be used as substitutes for essential elements (e.g., calcium replaced by lead, zinc by cadmium, and most trace elements by aluminum). NPs and MPs, small plastic pieces less than 1 and 10 μm in size, respectively,[7] can derive from plastic container degradation, during their production or stocking. These chemically inert plastic debris raise significant ecological and health concerns.[8,9] In particular, NPs and MPs in lung epithelium trigger cytotoxic effects, oxidative stress, inflammatory response, cell cycle arrest, upregulation of nuclear factor-κB, and disruption of the epithelial cell layer.[10] A number of toxicological tests are necessary to establish their reduced harm potential for smokers and to ensure protection of individual and public health from the adverse effects of potentially harmful exposures.[11] Cytotoxicity assays have been widely used to assess the toxicological impact of tobacco smoke[12] and of alternative tobacco products, including electronic cigarettes.[13–16] Since there is not a specific indication or protocol for toxicity evaluation of e-cig on human bronchial epithelial cells, we used a number of in vitro cytotoxicity assays to screen four different e-liquids with a high concentration of nicotine (15 mg/ml) in comparison with the 1R6F reference cigarette (University of Kentucky) using an air-liquid interface (ALI) exposure system. Moreover, we aimed to evaluate the production of reactive oxygen species (ROS) in the aerosol and intracellularly. Finally, we evaluated mitochondrial fitness, in order to assess the effect of aerosol exposure on cell metabolism.

2 | METHODS

2.1 | Nano and microplastic analysis

Analyses were carried out according to patent method described in Ferrante et al.[17] Briefly, after homogenization of samples by vortex, for each one, 10 ml was transferred to 50 ml transparent glass flask. Afterwards, 10 ml of nitric acid 65% was added, and mineralization of the samples was performed in an open vessel at 60°C for 24 h. After this, 10 ml ultrapure water and 10 ml of dichloromethane were added in each sample. Subsequently, samples were centrifuged at 4,000 rpm for 5 min. The solvent was dispersed on an aluminum and copper alloy stub with a diameter of 25 mm through nebulization by a nebulizer. After stubs were coated with gold, samples were ready to SEM-EDX analysis. The counting method was applied to an overall reading area within the stub for a total of 228 fields at magnification of 1,500X, corresponding to 1.0 mm^2. Micro-analytical acquisition for recognition of the particles containing only carbon and determination of particle size and counting was performed.

2.2 | Trace elements analysis

Aliquots of 1 ml of each e-liquid were digested with 3 ml of Nitric Acid 65%, Suprapur® for trace analysis (Carlo Erba Reagents, Milan, Italy) in a Microwave Ethos TC (Milestone, Sorisole, BG, Italy), equipped with pressurized Teflon vessels. The digestion was performed stepwise up to 200°C in 10 min (1,000 W), followed by a 15 min rest at 200°C (1,000 W). At the end of mineralization, the digested samples were transferred into graduated polypropylene tubes and diluted to 20 ml using Milli-Q water and filtrated through 0.45 μm nylon filters before analysis.

Trace elements were quantified with an Inductively Coupled Plasma–Mass Spectrometer (ICP-MS Elan DRCe, Perkin Elmer USA). The instrument was calibrated using the standard addition technique to minimize matrix effects, covering a concentration range from 0.5 to 10 μg/L, and a 25 μg/L concentration of Y as internal standard. Mono-elemental certified standards (1,000 mg/L) were purchased from CPAchem S.r.l. (Rome, Italy).

The limit of detection (LOD μg/L) were calculated by analyzing 10 acid extract blanks based on the mean ± 3 SD/mean ± 10 SD

criterion. They resulted as follows: Al < 4.4; As < 1.1; B < 18; Cd < 0.1; Cr < 0.5; Cu < 1.1; Fe < 6; Hg < 0.5; Mn < 1.5; Ni < 1.0; Pb < 0.5; Sb < 0.5; Se < 0.5; V < 1.1.

As quality controls, each sample was spiked at 10 μg/L before digestion. The recoveries calculated are in the range 91–118%.

2.3 | Cell culture and ALI exposure

Human primary bronchial epithelial cells (NHBE, ATCC® PCS-300-010™) were cultured in Airway Epithelial Cell Basal Medium (PCS-300-030; ATCC, VA, USA) plus Bronchial/Tracheal Epithelial Cell Growth Kit (PCS-300-040; ATCC, VA, USA) (50 U/ml penicillin and 50 mg/ml streptomycin) at 37°C, 5% CO_2 in a humidified atmosphere. Then, cells were seeded in 12 mm Transwells® inserts (Corning Incorporated, NY, USA) at a density of 1.75×10^5 cells/ml sustained by 1 ml of medium in the basal compartment of each well and 0.5 ml in the apical compartment of each insert (Figure S1), 48 h prior to exposure. When the cells reached 80% confluency, the apical medium was removed from each insert, and two inserts per test product were transitioned to the perspex exposure chamber[16,18,19] (Figure S1) with 20 ml of DMEM-high glucose (DMEM-hg) in the basal compartment in order to perform the air-liquid interface (ALI) exposure, as previously described.[15,20] This exposure method is the most physiologically relevant for bronchial epithelial cell lines exposing them to all fractions and components of smoke/aerosol.[19] For each smoking/vaping exposure, one chamber was connected to the LM4E port without the device so as to expose NHBE cells to laboratory air filtered by a Cambridge Filter Pad at the same regime (AIR control). Moreover, two negative controls, consisting of a seeded insert with media submerged (INC) and a seeded insert without apical media (ALI) in the incubator, and one positive control with 1 ml apical and 2 ml basal sodium dodecyl sulfate (SDS) at 350 μM were included for each set of exposure. After each exposure, the inserts were transferred from the chamber to a clean well plate, adding 1 and 0.5 ml of supplemented medium, respectively, at the basal and apical side for 24 h of recovery period. The recovery period was not performed for Neutral Red Uptake (NRU) Assay in live and xCELLigence Real-Time Cell analysis.

2.4 | Test products and exposure regimens

For the exposure of NHBE cells, we used a standardized experimental tobacco cigarette, 1R6F (University of Kentucky), and four electronic cigarette liquid samples (hereinafter referred to as "e-liquids"), chosen as they are different tobacco flavored products, widely sold in Italy, tested in our laboratories: "DolceBacco" No. 20 containing 15 mg/ml of nicotine (A), "RedBacco" No. 19 containing 15 mg/ml of nicotine (B), "Deciso" No. 22 containing 15 mg/ml of nicotine (C), and "Otello" No. 23 containing 15 mg/ml of nicotine (D). All tested e-liquids are produced and marketed in Italy by Dreamods S.r.l. (Floridia, SR, Italy). Moreover, a solution containing only PG/VG (1:1) and a PG/VG (1:1) with nicotine 15 mg/ml were used as controls. The e-liquids and PG/VG solutions (with and without nicotine) were tested using the device Aspire Zelos mounting the "Nautilus 2" atomizer, equipped with a 1.6 Ohm Coil and set at 10-W power. Aspire Zelos is a button-activated e-cigarette with an internal 2,500 mAh lithium battery with a variable wattage from 1 to 50 W. The "Nautilus 2" is a 2 ml top-fill tank which has the possibility to mount coils between 0.7 and 1.8 Ohm. It has an airflow adjuster at the base of the tank which can be turned to adjust the airflow with five holes which can be more or less closed to reduce the air flow and tighten the draw. We regulated the airflow adjuster to three open holes, capable of ensuring a discreet air flow during the puffs. To expose NHBE cells to five puffs of undiluted smoke from 1R6F was used the "Health Canada Intense" (HCI) regimen, accredited under ISO/TR 19478-2:2015,[21] which ensures a 55 ml, 2 s duration bell shaped profile, puff every 30 s (55/2/30) with filter hole vents blocked, and e-cigarette aerosol was produced under the "CORESTA Reference Method n. 81" (CRM81) regimen (55 ml puff volume, drawn over 3 s, once every 30 s with square shaped profile), accredited into ISO 20768:2018.[22] The puff number for each product was established according to nicotine dose delivered from 1R6F (data not shown), in order to have a similar or greater nicotine delivery of an entire 1R6F tobacco cigarette, which is able to deliver 0.45 μg/ml of nicotine per puff, measured by UPLC-MS in 1R6F cigarette bubbled culture media. Otherwise, e-cig with the e-liquids containing 15 mg/ml of nicotine were able to release 0.30 μg/ml of nicotine per puff with the device and settings used in the study. We therefore exposed cells to a quantity of nicotine from e-cig greater than that of a whole 1R6F cigarette with nine puffs by HCI regimen (4.05 μg/ml), that is, 20 puffs of e-liquids by CRM-81 regimen (6 μg/ml), to perform a stronger exposure to aerosol and to reproduce an intense vaping session. Moreover, we exposed cells to the same puff number ($n = 20$) by CRM-81 regimen of air as control (AIR control).

2.5 | Smoke and aerosol generation

1R6F reference cigarettes (Center for Tobacco Reference Products, University of Kentucky) were used for smoke exposure. Cigarettes were conditioned at 22 ± 1°C and 60 ± 3% of relative humidity for at least 48 h according to ISO 3402:1999 guidelines. LM1 smoking machine (Borgwaldt KC GmbH, Hamburg, Germany) was used to smoke 1R6F cigarette (Figure S2) and to deliver undiluted smoke to exposure chambers containing cells. Instead, e-liquids were vaped by LM4E vaping machine (Borgwaldt KC GmbH, Hamburg, Germany) (Figure S2); also, it is capable of delivering undiluted aerosol to exposure chambers with cells. To evaluate the ROS production by aerosol, we connected the machines to glass impinger containing medium and bubbled the e-cig aerosol by HCI regimen for 10 to 60 puffs. In order to perform the ALI exposure of cells, smoking and vaping machines were connected to the exposure chambers containing cells, which are maintained at 37°C in a total visibility incubator (SI60 Incubator; Cole-Parmer, Staffordshire, UK) throughout the duration of the exposure (Figure S3).

2.6 | ROS production by aerosol

The e-liquids (A–D), the PG/VG, and PG/VG containing nicotine were tested to assess the production of ROS in aerosol by a simple "cell-free" assay.[23] An increasing number of puffs from each of the e-liquids mentioned above were bubbled in DMEM culture medium, FBS-free, with 2′,7′-Dichlorofluorescin diacetate (DCF-DA), and the fluorescence was measured by a fluorimeter by calculating the amount of H_2O_2 equivalents formed with respect to a standard curve obtained with increasing concentrations of H_2O_2. This experiment was conducted for different numbers of puff (10–60), and laboratory-air bubbled media was used as basal control. The experiments were conducted in triplicate.

2.7 | Assessment of cytotoxicity by RTCA

After the exposure to smoke, aerosol, and air, cell viability was evaluated using RTCA. At the end of each exposure, cells were washed twice with PBS, trypsinized (0.25% trypsin), counted, and resuspended in supplemented RPMI-1640. Then, cells were seeded in E-16 xCELLigence plate (Agilent, CA, USA) at a density of 3×10^3 cells/ml per well. The plates were subsequently incubated at 37°C, 5% CO_2 for 30 min in order to allow cell settling. Real-time cell proliferation analysis was performed using the xCELLigence RTCA DPsystem (Agilent, CA, USA). The xCELLigence is designed for monitoring cell adhesion and growth. The system exploits microplates with gold electrodes on the bottom of the wells, such that an electric potential is applied across wells. Therefore, adhering cells to a well reduce the degree of electrons able to flow freely across the established potential (electrical impedance).[24] Real-time changes in electrical impedance are measured by xCELLigence analyzer and expressed as "cell index," defined as (Rn-Rb)/15, where Rb is the back-ground impedance and Rn is the impedance of the well with cells. The background impedance was measured in E-plate 16 with 100 µl medium (without cells) after 30 min incubation period at room temperature. Cell proliferation was monitored every 20 min for 71 h.

2.8 | Assessment of cytotoxicity by HCS system

Exposed NHBE cells were washed twice with PBS and detached from the inserts with trypsin (0.25%). Cells were seeded in a CellCarrier™-96384 well (PerkinElmer) at a density of 13×10^3 cells/ml and incubated at 37°C, 5% CO_2 for 24 h. Next, cells were labeled with 0.05 g/L NR dye and 2 droplets/ml NucBlue™ (Invitrogen, ThermoFisher Scientific, USA) in UltraCULTURE™ medium and incubated for 3 h at 37°C, 5% CO_2 in a humidified atmosphere for staining. After the staining, medium with dyes was removed from each well, and cells were washed twice with PBS. Then, 200 µl of fresh supplemented RPMI-1640 medium was added in each well. The plate was read under confocal conditions using the 20× long WD objective by High Content Screening (HCS) analysis (PerkinElmer Operetta High-Content Imaging System). Exposed NHBE cells were monitored every 1 until 24 h and then every 4 until 71 h. All images were analyzed using Harmony high-content imaging and analysis software (PerkinElmer, MA, USA). Final output values from the analysis are expressed as mean fluorescence intensity (MFI) percentage of control per well. Live cell viability curves were generated for each tested product.

2.9 | Assessment of mitochondrial potential

In addition to cytotoxicity and intra-cellular reactive oxygen species (ROS) production, we also evaluated other parameters using HCS: cell morphology and mitochondrial potential. All experiments were performed in triplicate, and the results were collected and analyzed by statistical analysis. Mitochondrial potential was assessed by the cationic dye JC-1, which exhibits potential-dependent accumulation in mitochondria.[25] Briefly, after smoking and aerosol exposure, cells were detached and seeded in a 384-well multiplate (Cell carrier ultra) at a density of 3×10^3 cells. After 24 h, cells were incubated with media containing dye (1 µg/ml) for 1 h, and with SYTO Deep Red-NucBlue Nucleic Acid Stain (excitation/emission of 652/669 nm), which is able to enter into the live/dead cells and bind to nucleic acids. After incubation with dyes, cells were washed twice and then read under confocal conditions using the 20× long WD objective by High Content Screening (HCS) analysis system (PerkinElmer Operetta High-Content Imaging System) for 72 h.

2.10 | Statistics

Comparison of ROS cell-free results was performed by using ANOVA followed by Bonferroni's correction for multiple testing. Comparisons among the tested products of RTCA, NRU-HCS, and Nuclei morphology were analyzed by fitting a repeated measure mixed model followed by Tukey's test to perform multiple comparisons. Moreover, comparisons of JC1 results were analyzed by fitting a repeated measure mixed model followed by Tukey's test adjustment for multiple comparisons. Data were expressed as mean ± standard error (SE). All analyses were considered significant with a p value of less than 5%. We analyzed and plotted the results using GraphPad Prism version 8 (GraphPad Software, San Diego, California, USA, www.graphpad.com).

3 | RESULTS

3.1 | Nano-microplastics and trace elements

The analysis of NPs and MPs showed no plastic debris smaller than 10 µm in size in any of the analyzed samples. The analysis of 14 trace elements was performed in four samples of e-liquid (A–D), and results are shown in Table 1. Metallic elements considered systemic toxicants

in all their chemical forms (As, Hg, and Pb) or only some (inorganic As and Cr [VI]) were found below the limit of detection in all the analyzed samples, with the exception of As in samples A and B where we found very small amount, 3.3 and 2.3 μg/L, respectively. For some elements considered essential, and respect of which there is a very narrow range of concentrations between beneficial and toxic effects, we found concentrations below the LOD or close to it in the case of Al and Ni in A and B and Se in all the analyzed samples. Fe has the higher concentration, in the range from 75 to 113 μg/L, followed by B, in the range from 19 to 32 μg/L. Sb has not biological function and was found below LOD in all analyzed samples.

3.2 | Effects of e-liquids on ROS formation in a cell-free based assay

Our results on ROS formation demonstrated that PG/VG (1:1) does not generate ROS in the absence of nicotine (Table 2). The vaporization of a PG/VG formulation containing nicotine (15 mg/ml) instead generates the production of ROS in proportion to the number of puffs. This result is consistent with previous data demonstrating that nicotine pyrolysis products can generate ROS. Products A–C do not generate ROS in these experimental conditions, while product D shows a ROS production directly proportional to the number of puffs (Figure 1). It is interesting to note that the ROS production of the latter product (D) is however significantly lower than only PG/VG with nicotine for 10 puffs ($p = 0.0004$), 20 puffs ($p = 0.004$), 40 puffs ($p < 0.0001$), and 60 puffs ($p < 0.0001$). The measurements were performed in triplicate.

TABLE 1 Concentrations (μg/L) of trace elements in e-liquid (A–D)

Trace element	Sample A	Sample B	Sample C	Sample D
Al	10.1	12.3	<4.4	8.6
As	3.3	2.3	<1.1	<1.1
B	24	32	19	26
Cd	<0.5	<0.5	<0.5	<0.5
Cr	<0.5	<0.5	<0.5	<0.5
Cu	<1.1	<1.1	<1.1	<1.1
Fe	113	102	75	112
Hg	<0.5	<0.5	<0.5	<0.5
Mn	<1.5	<1.5	<1.5	<1.5
Ni	1.6	2.3	<1.0	<1.0
Pb	<0.5	<0.5	<0.5	<0.5
Sb	<0.5	<0.5	<0.5	<0.5
Se	1.2	0.9	0.7	<0.2
V	<1.1	<1.1	<1.1	<1.1

3.3 | Effects of e-liquids on cell viability evaluated by RTCA and neutral red uptake in live imaging analysis (NRU-HCS)

For each product (A–D), we assessed the viability by RTCA vs. 1R6F cigarette smoke. Figure 2 shows the results for each product

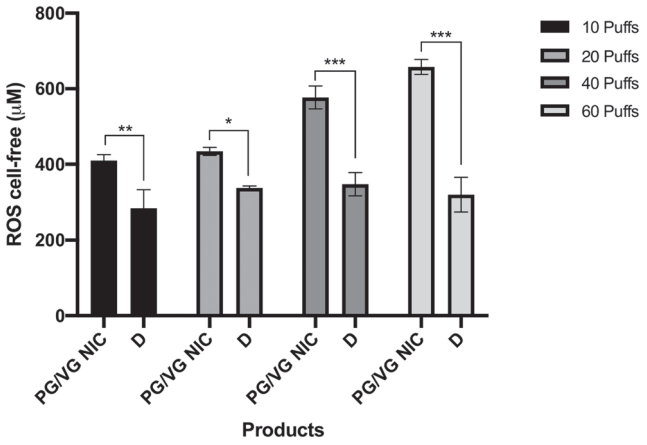

FIGURE 1 Comparison of ROS cell-free production from aerosol of PG/VG with nicotine and e-liquid D. Data are presented as the mean ± standard deviation and referred to μm of H_2O_2. $^{*}p < 0.01$; $^{**}p < 0.001$; $^{***}p < 0.0001$

TABLE 2 ROS values in the cell free assay system

	Conditions	Puff number			
		10	20	40	60
Product ID	Air	n.d.	n.d.	n.d.	n.d.
	PG/VG	n.d.	n.d.	n.d.	n.d.
	PG/VG + Nicotine 15 mg/ml	410.1 ± 15.20	434.24 ± 10.58	576.77 ± 30.17	657.7 ± 19.77
	A (Dolce Bacco)	n.d.	n.d.	n.d.	n.d.
	B (Red Bacco)	n.d.	n.d.	n.d.	n.d.
	C (Deciso)	n.d.	n.d.	n.d.	n.d.
	D (Otello)	283.5 ± 49,33	337.15 ± 5.61	347,37 ± 30,94	319,71 ± 45,99

Note: Data are presented as the mean ± standard deviation and referred to μm of H_2O_2. Values not detectable by the fluorometer are indicated as n.d. (not detectable).

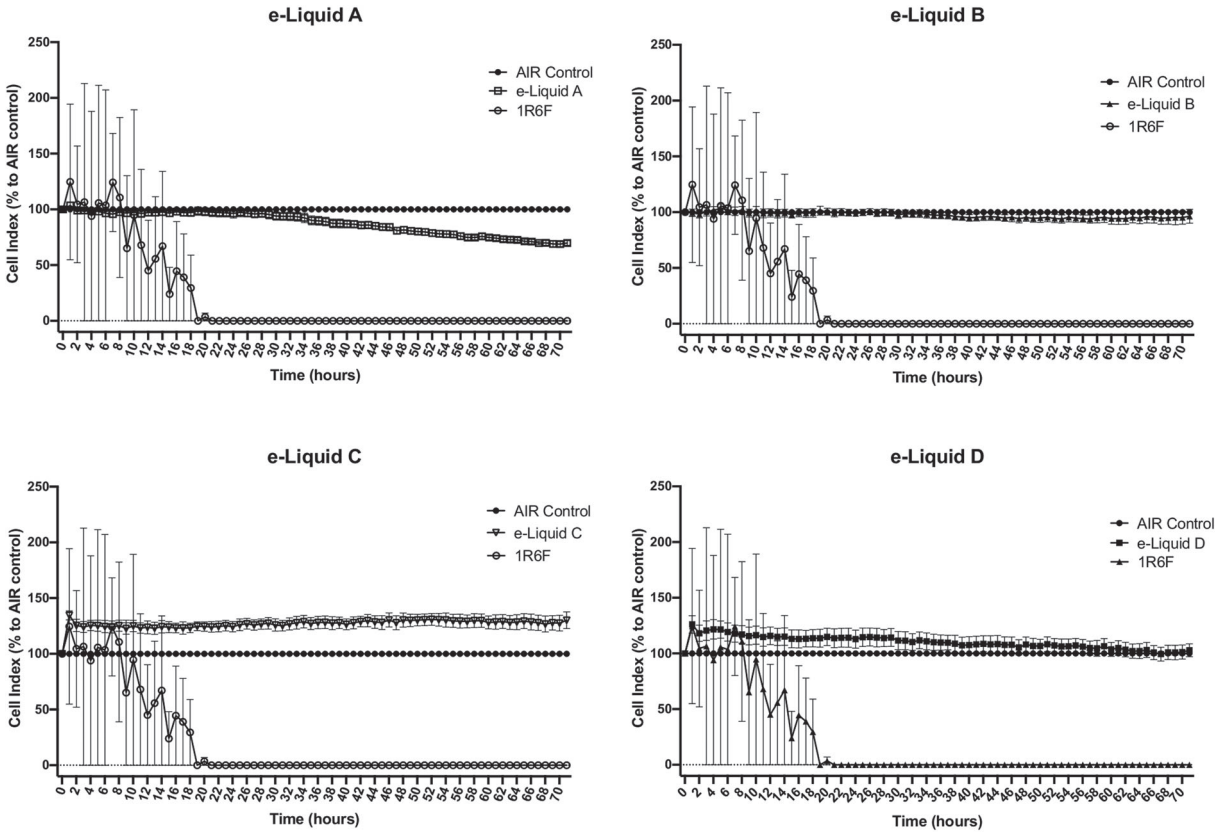

FIGURE 2 xCELLigence data reported as a percentage of the AIR control indicated as 100% for all observation times. The values indicated in the graph refer to mean ± SEM of the electrical resistance (Cell index) which is directly proportional to the number of cells

monitored for up to 71 h using xCELLigence technology. 1R6F cigarette smoke showed a surge in cell-index, and therefore cell proliferation, in the first 6–8 h and then collapsed to 100% mortality already at 19 h; 1R6F growth curve differs significantly from AIR Control and the four e-liquids ($p < 0.0001$). The e-liquids aerosol stimulation showed that none of the tested products had significant toxicity in the first 24 h of exposure and not even at 71 h, as for AIR exposure of cells. Particularly, no significant differences were observed for e-liquids A, B, and D compared to AIR Control ($p > 0.05$); instead, e-liquid C increased the cell index, showing a significant difference than AIR control ($p = 0.015$). The data relating to cell viability were further confirmed using a test (neutral red uptake) which considers cellular metabolic activity. In this set of experiments (Figure 3), product A (Dolce Bacco) showed a transient reduction of viability in the first 24 h from the exposure, subsequently recovering to reach levels of vitality similar to AIR exposure for up to 71 h. A similar trend was observed for product B. Products C and D showed a minor reduction of cell viability by NRU-HCS assay in the early hours and a recovery of cell viability starting approximately from 24 h. The cell growth curves of all e-liquids were significantly different from the 1R6F cell growth curve ($p < 0.0001$), which showed a reduced cell viability throughout the 71 h of observation.

These results are supported by the cell morphology (Figure 4) which appears to be more irregular in cells treated with the reference cigarette than with e-liquids A, B, C, or D ($p < 0.0001$). Products A and B showed reduced nuclear roundness compared to AIR control ($p < 0.0001$), but this reduction is less than 1R6F. Instead, e-liquids C and D showed no difference in nuclei roundness compared to AIR Control.

3.4 | Effects of e-liquids on cellular metabolism

These experiments aiming at evaluating the mitochondrial function by studying the depolarization of the mitochondrial membrane with the JC1 fluorescent probe have shown that all the products tested (A–D) did not cause loss of mitochondrial function (Figures 5 and S4–S8).

Under these experimental conditions, all products tested showed significantly better mitochondrial function than the reference cigarette 1R6F ($p < 0.0001$). The mitochondrial function values for the reference cigarette show a dramatic and rapid drop in mitochondrial potential compared to AIR control and all e-liquids ($p < 0.0001$).

4 | DISCUSSION

The tested e-liquids showed a good safety profile in terms of absence of micro- and nano-plastics despite the plastic packaging, highlighting

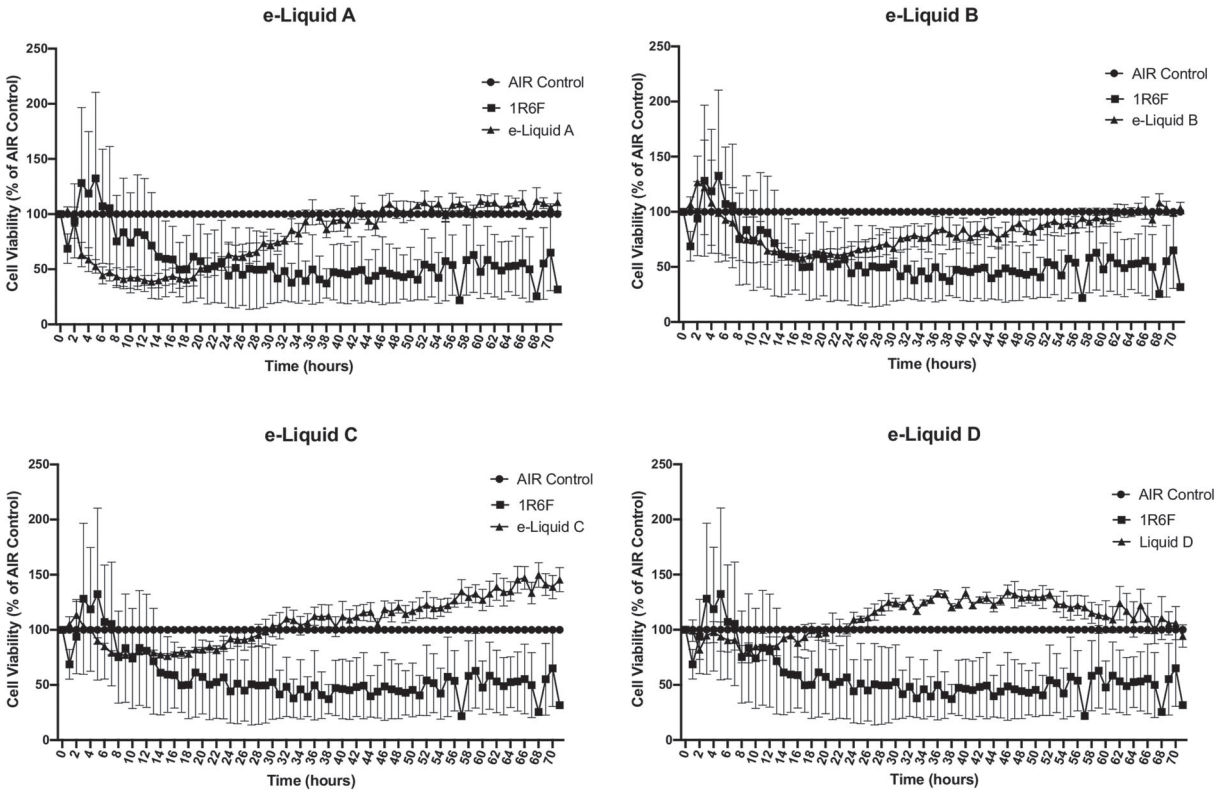

FIGURE 3 Neutral red uptake in live imaging analysis (NRU-HCS). The assay relies on the ability of living cells to incorporate and bind neutral red, a weak cationic dye, in lysosomes. As such, cytotoxicity is expressed as a concentration-dependent reduction of the uptake of neutral red after exposure to the aerosol from eliquids under investigation (A–D) and smoke from reference cigarette (1R6F). Data are shown as mean ± SEM percentage of AIR control

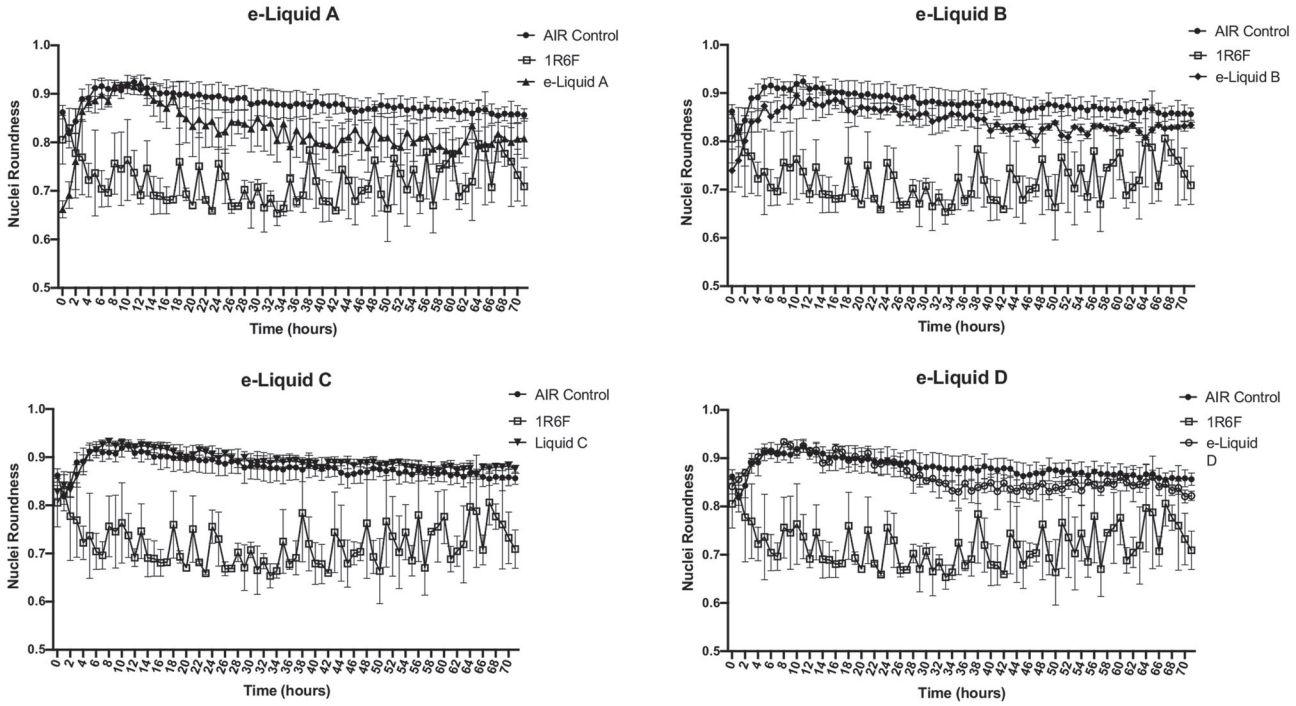

FIGURE 4 Morphological analysis of the nuclei in live imaging analysis. The value is obtained by dividing the length by the width and the ratio indicates the regular rounded shape the closer the value is to 1. Data are shown as mean ± SEM

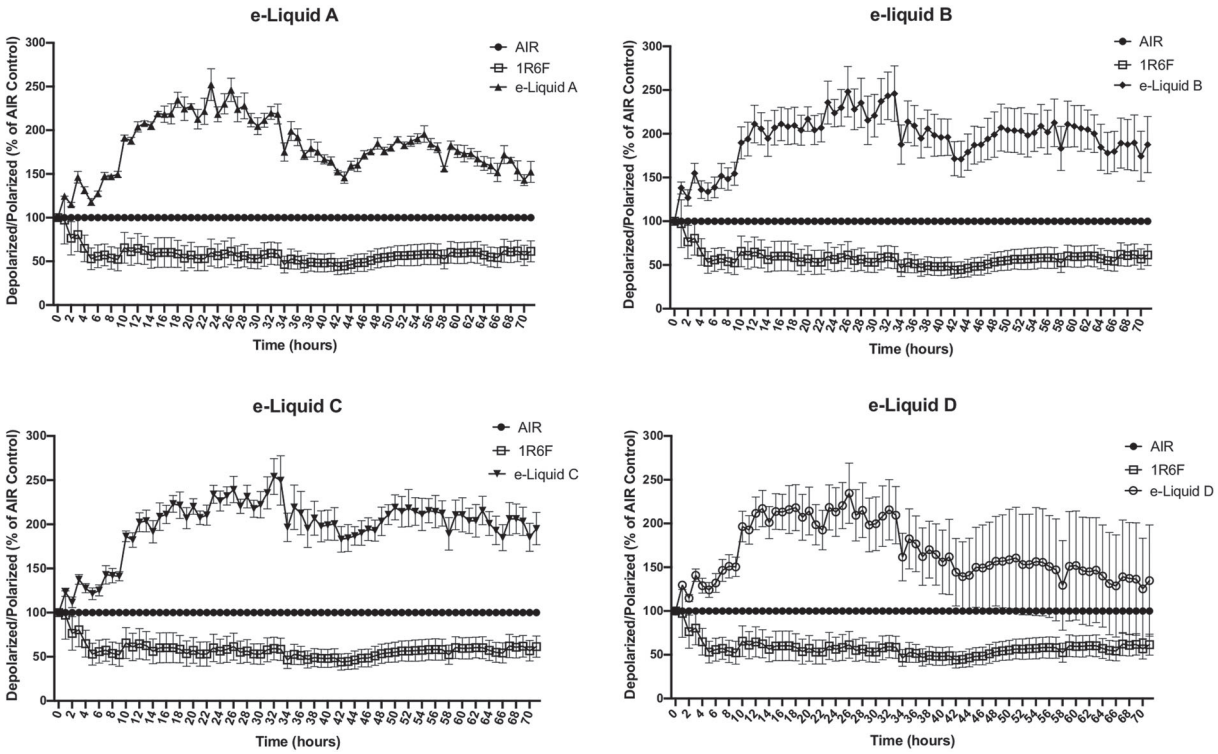

FIGURE 5 The data shown in the graph are obtained by the ratio of the fluorescence values at different wavelengths of the JC-1 probe. The values are expressed as mean ± SEM percentage of AIR control

a good quality of the material used and production processes, transport, and storage of the products before marketing. In addition, the liquids showed a very low concentration of trace elements, confirming their safety with respect to the contaminants of products intended for human consumption. Among the metallic elements, only the aluminum (Al) and iron (Fe) showed values significantly above the LOD. As far as concern Al, within a certain dosage, it is not considered a toxicant for human. In particular, the FDA has determined that Al used as food additives and medicinal such as antiacids, astringents, buffered aspirin, food additives, and antiperspirants are generally safe. Moreover, the United States Environmental Protection Agency (EPA) has recommended a Secondary Maximum Contaminant Level (SMCL) of 0.05–0.2 mg/L for aluminum in drinking water.[26] In tested e-liquids, we found concentrations of 0.0086–0.0123 mg/L for Al, therefore levels from 5 to 20 times lower than the maximum limits for drinking water. The last metallic element with considerable concentrations, compared to the other elements, was Fe. Concentrations of iron in drinking water are normally less than 0.3 mg/L,[26] and therefore about three to four times higher than the concentrations found in the e-liquids tested in this study (0.075–0.1 mg/L). As non-metallic elements founded at measurable concentrations, we found the boron (19–32 μg/L). It is important to point out that borate compounds are ingredients of domestic washing agents; therefore, these residues could be contaminants coming from the cleaning products of machines and containers. But more importantly, we must consider that the concentrations of boron in drinking water in the world have wide ranges, largely dependent on the source of the drinking water, but WHO judged that the concentration of boron in drinking water needs to be below 0.5 mg/L[26] (possibly between 0.1 and 0.3 mg/L). Therefore, the concentrations detected in the e-liquids tested (0.019–0.032 mg/L) are considered widely safe for human consumption.

Consistently, all tested e-liquids showed an excellent safety profile and low toxicity even after vaporization with an e-cig, albeit with a slightly different profile from one liquid to another. Although the PG/VG solution with nicotine produces a certain amount of ROS during the vaporization by e-cig, possibly due to the pyrolysis effect of nicotine,[27–30] products A–C did not produce ROS under the conditions tested in the cell-free system assay, suggesting that the aromatic part of the products can offset the production of ROS. It is therefore possible to hypothesize that the formulation of the flavors contained in the products tested may act as a scavenger with antioxidant effect[31–33] able to balance the production of ROS from nicotine pyrolysis.[27–30] Just as an example, product A is described as a vanilla flavored tobacco from the manufacturer. Vanillin, a phenolic compound widely used in foods, beverages, cosmetics, and drugs, has been reported to exhibit multifunctional effects, including some antioxidant effect.[31–33] Product D showed an increase in ROS in the same system compared to the other products but still significantly lower than PG/VG with nicotine alone. In this regard, it is necessary to review the composition of the aromatic part of this product in order to reduce the production of ROS while maintaining its sensory

properties for the consumer. Interestingly, when used with the settings of resistance and power detailed in methods, all the products did not show significant and permanent alterations of the cellular metabolism even in the longest periods of observation.

The cytotoxic effect induced by the aerosol produced with these e-liquids is greatly reduced compared to that induced by the smoke of a classic cigarette. These data were confirmed in our model by three different approaches: the RTCA analysis, the NRU analysis by HCS, and the morphological analysis of the nuclei, observing the cells up to 72 h post-exposure. RTCA showed an excellent viability and ability to cellular proliferation potential of almost all the tested products. Cells exposed to products B and D had a viability similar to the cells exposed to laboratory air (AIR). Product D even showed an increase in cell viability than AIR control. Probably, the flavoring mixture contained in product D was not toxic for NHBE cells and positively influenced their adhesion and proliferation. Instead, e-liquid A was the only product to show a decrease in viability from the 36th hour onwards, but this diminished vitality was not comparable to zero viability after exposure with 1R6F smoke. A point to emphasize regarding the RTCA analysis was the large error bars observed for 1R6F values. We observed microscopically that cells exposed to 1R6F exhibited tar residues on their surface, and this may have adversely affected cell adhesion on xCELLigence microplates producing high variability in the results. Using the NRU with HCS analysis, we were able to monitor cell viability up to 72 h post-exposure. We observed an initial decrease in cell viability followed by recovery for all products tested. Also, for NRU analysis, product A showed a greater decrease in cell viability than the other products. Maybe acute exposure to product A aerosol affected cell viability but still to a lesser extent than cigarette smoke.

Mitochondrial potential was also assessed by the cationic dye JC-1, which exhibits potential-dependent accumulation in mitochondria, indicated by a fluorescence emission shift from green (~525 nm) to red (~590 nm). Mitochondrial depolarization is indicated by a decrease in the red/green fluorescence intensity ratio. The potential-sensitive color shift is due to concentration dependent formation of red fluorescent aggregates. The ratio of green to red fluorescence is dependent only on the membrane potential. Comparative measurements of membrane potential allow to determine the percentage of mitochondria able to respond to an applied stimulus and to highlight the mitochondrial depolarization occurring in the early stages of apoptosis.

The results of the study indicate lower metabolic perturbation at mitochondrial level, no production of reactive oxygen species, and a substantially reduced cytotoxic effect of e-liquid aerosol compared to cigarette smoke. Under normal condition of use and with the power settings recommended by manufacturers, the vaping products under investigation proved to be significantly less harmful to human cell systems compared to conventional cigarettes.

A possible limitation of this study may be represented by the lack of experiments with different device settings, but for which do not exist standardization so far. To this regard, different conditions of power settings and resistances could provide different results from those reported here. Another limitation is the lack of knowledge of the chemical flavors composition used in the tested liquids (all tobacco flavored e-liquids), which could have allowed further understanding of the different results obtained. Such results would have provided important informations for manufacturers of e-liquids allowing them to perform a top-down analysis in order to identify the most dangerous components of each liquid reformulating their products in a safer way for consumers.

5 | CONCLUSIONS

Our results support the reduced harmful potential of e-cigs relative to tobacco cigarettes in an in vitro model of normal human bronchial epithelial cells and support the use of electronic cigarettes as a viable strategy in harm reduction strategies for smokers.

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CONFLICT OF INTEREST

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. In relation to his recent work in the area of respiratory diseases, clinical immunology, and tobacco control, Riccardo Polosa has received lecture fees and research funding from Pfizer, GlaxoSmithKline, CV Therapeutics, NeuroSearch A/S, Sandoz, MSD, Boehringer Ingelheim, Novartis, Duska Therapeutics, and Forest Laboratories. Lecture fees from a number of European EC industry and trade associations (including FIVAPE in France and FIESEL in Italy) were directly donated to vaper advocacy no-profit organizations. Riccardo Polosa has also received grants from European Commission initiatives (U-BIOPRED and AIRPROM) and from the Integral Rheumatology & Immunology Specialists Network (IRIS) initiative. He has also served as a consultant for Pfizer, Global Health Alliance for treatment of tobacco dependence, CV Therapeutics, Boehringer Ingelheim, Novartis, Duska Therapeutics, ECITA (Electronic Cigarette Industry Trade Association, in the UK), Arbi Group Srl., Health Diplomats, and Sermo Inc. Riccardo Polosa has served on the Medical and Scientific Advisory Board of Cordex Pharma, Inc., CV Therapeutics, Duska Therapeutics Inc, Pfizer, and PharmaCielo. RP is also 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 (CoEHAR) at the same university, which has received support from Foundation for a Smoke Free World to conduct eight independent

investigator-initiated research projects on harm reduction. Riccardo Polosa is currently involved in a patent application concerning an app tracker for smoking behavior developed for ECLAT Srl. RP is also currently involved in the following pro bono activities: scientific advisor for LIAF, Lega Italiana Anti Fumo (Italian acronym for Italian Anti-Smoking League), the Consumer Advocates for Smoke-free Alternatives (CASAA) and the In-ternational Network of Nicotine Consumers Organizations (INNCO); 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. The other authors have no relevant financial interests to disclose.

AUTHOR CONTRIBUTIONS

M.C. and G.L.V. conceived the study and were in charge of overall direction and planning; G.L.V. and M.F. verified the analytical methods; A.D., R.E., P.Z., C.C., and R.Pu. carried out the experiments; R.E., C.C., and G.C. contributed to the interpretation of the results; S.R. and G.A.M. administered the project fundings; M.C. wrote the manuscript with support from A.D. and R.E.; G.L.V. and G.R. contributed to the final version of the manuscript; R.Po. supervised the project. All authors discussed the results and contributed to the final manuscript.

DATA AVAILABILITY STATEMENT

The datasets generated and/or analyzed during the current study are available from the corresponding author on reasonable request.

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SUPPORTING INFORMATION

Additional supporting information may be found in the online version of the article at the publisher's website.

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