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E-liquid Base Ingredients Induce Oxidative Distress and Epithelial Barrier Disruption in the Co-Culture Model of A549 and THP1 Cells

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ABSTRACT

Electronic cigarettes heat and aerosolize an e-liquid composed from propylene glycol (PG) and glycerin (VG). These bases enter the respiratory tract where tight junction (TJ) proteins, which regulate paracellular permeability, may affect alveolar function. The aim was to evaluate TJ alteration by exposure to PG at 30%, VG at 70% and their mixture PG-30: VG-70 in the A549 cell line in co-culture with THP1 cells. We evaluated:

1. Cell viability by MTT (3-(4,5-dimethylthiazol-2yl)-2,5-diphenyltetrazol bromide) and neutral red;
2. Biomarkers of oxidative stress on lipid (malondialdehyde, MDA) and protein damage (carbonyl assay) and lipid droplet formation by oil red staining;
3. Epithelial integrity by transepithelial electrical resistance (TEER), and
4. TJ expression (occludin, claudin-1, claudin-2 and claudin-4) by Western blot.

Lipid and protein oxidation increased after all treatments, but lipid droplets increased only after mixing. TEER decreased after treatments, suggesting increased permeability. Lipid and protein oxidation increased after all treatments, but lipid droplets increased only after mixing. TEER decreased after treatments and TJ proteins showed compensatory changes expressed by increases in claudin-1, -4 and occludin and decreases in claudin-2. Exposure to bases induced oxidative stress leading to protein and lipid oxidation, accumulation of lipid droplets and disruption of TJ assembly.

Keywords: E-cigarettes; E-liquid bases; Propylene glycol; Glycerin; Vaping; Tight junction proteins, Oxidative stress

Abbreviations: TEER: Transepithelial Electrical Resistance, PG: Propylene Glycol, VG: Vegetable Glycerin, TJ: Tight junction.

Introduction

Electronic cigarettes, also known as "e-cigarettes", "e-cigs", "vapers", and "electronic nicotine delivery systems", among others, are battery-powered devices that produce an aerosol by heating and subsequently cooling a liquid, also known as an e-liquid. E-liquids have an elemental composition of propylene glycol (PG) and vegetable glycerin (VG) in different proportions; generally, deliver nicotine, flavorings, and other substances [1,2]. Initially, vapers were used as an alternative to cigarette smoking in prohibited places and publicized as an alternative to quitting tobacco smoking. It is essential to know that although the toxic components in the vapers are lower than in combustion cigarettes, it does not imply that they are less harmful. It becomes a new health problem in vapers that never smoked cigarettes and in those who become dual users, those who vape and smoke cigarettes because they could not quit smoking [2]. An association has been reported between vaping as a cause of multiple adverse reactions, the most important being respiratory injuries such as endothelial function damage, acute eosinophilic pneumonia, diffuse alveolar hemorrhage, primary spontaneous pneumothorax, organizing pneumonia, acute respiratory distress syndrome, e-cigarette or vaping product use-associated lung injury (EVALI) and hypersensitivity pneumonitis; cardiovascular injury such as blood disorders, endocarditis, myocardial infarction and increase in cardiovascular hemodynamic measure; immune system and adverse mental health [3-6]. It is known that the PG:VG proportion can influence the delivery of the substances and therefore, aerosol toxicity [1,2]. The e-liquids have a usual base comprised of a PG:VG proportion (30:70), which generates thicker and milder-tasting aerosols with large amounts of vapor. The Food and Drug Administration (FDA) considers both PG and VG to be "generally recognized as safe" [7]. However, the GRAS designation only applies to dermal application or ingestion and does not address inhalation-aerosol exposure to these products or their thermal degradation products. When the e-liquid passes the resistance of the e-cigs, basic substances are not completely heated, regardless of the generation of the e-cigarette device. Thus, PG and VG are detected in the final products along with polycyclic aromatic hydrocarbons (PAHs; anthracene, phenanthrene, 1-methylphenanthrene, pyrene, acenaphthylene, fluorine, naphthalene, 2-methylnaphthalene, 1-methyl naphthalene, 2-chloronaphthalene, and so on), volatile compounds such as epoxides (propylene oxide, glycidol or 2,3-epoxy-1-propanol), carcinogenic carbonyl compounds (formaldehyde, acetaldehyde, and acrolein), metals (Cr, Cu, Cd, Ni, Pb, As, Mn, Zn, Ag, Sb, Tl, Al, and Sn), nicotine derivatives, phthalates, and particles that are 2.5 microns or less in diameter (PM2.5), among others [8-13]. Evaluation of the aerosol dynamics generated by vaping has shown that ultrafine droplets or aerosols can be deposited along the respiratory tract and reach the alveoli. It does not matter whether vaping is active or passive [14,15]. Oxidant and inflammatory responses, in cultured cells, have been reported from exposure to e-liquid and aerosols produced by e-cigs. Imbalanced oxidative stress involves damage to all essential biocomponents of proteins, DNA and membrane lipids and can lead to cell death, cellular aging, and the onset of various types of disease [16-18]. However, few studies have focused on the effect of the bases, which vary the VG/PG ratio, and even use one of these substances or their proportion as a control for the effects they study without considering that the bases already induce an effect. Without heating and vaporization, the biomolecular and cellular mechanisms of injury caused by e-liquid compounds are not fully understood.

Therefore, it is necessary to evaluate the toxicological potential and independent cellular damage mechanisms of e-liquid bases and additive exposure. Vaping aerosols come into direct contact with the lung epithelium, which must maintain its integrity. Tight junction (TJ) proteins, primarily responsible for ion and water regulation, transport proteins and lipids across the paracellular space (between the apical and basolateral domains), establishing cell polarity and signaling. TJ proteins are a complex group of proteins that involve occludin, the claudin family, Junctional adhesion molecules (JAMs), cingulin, and the zonula occludens (ZO) family [19,20]. The co-culture is useful for studying natural interactions between populations, improving the culture cell success of populations, or establishing cell behavior [21]. We used a co-culture cell model to evaluate the natural interactions between the alveolar epithelial population (A549, adenocarcinomic human alveolar basal epithelial cell) and the peripheral monocytic population (THP1, peripherial monocyte isolated from an acute monocytic leukemia patient) due to the proximity to the bloodstream, hoping to observe their effects by the extracellular environment. Thus, this work aimed to evaluate oxidative stress through protein and lipid damage and epithelial barrier disruption by claudins 1,2,4 and occludin in a cell co-culture model with A549 and THP1 cell lines.

Materials and Methods

Chemicals

The chemicals purchased from Sigma Aldrich (St. Louis, MO, USA) were: MTT (3-(4,5-Dimethylthiazol-2yl)-2,5-diphenyltetrazole Bromide, M5655), oil red (1.02419.0250), MDA (malondialdehyde), MPI (methyl-2-phenylindole, 8.05797), DNPH (2,4-dinitrophenylhydrazine, DI149), penicillin- streptomycin-neomycin (P4083), glutamine (G7513) and Claudin-2 (SAB4503544). Sodium pyruvate (S8636), Fetal Bovine Serum (FBS, S00ND1002), from Life Technologies Gibco (N.Y., USA). Propylene glycol (U510-07) and glycerin (1435-500) from J.T. Baker (Phillipsburg, N.J., USA) and Meyer (Cd. Mexico, Mex), respectively. The chemicals purchased from Santa Cruz (Dallas, TX, USA) were Claudin-1 antibody (Sc-166338), Claudin-4 antibody (Sc-25712), occludin antibody (Sc-25712), and GADPH (Sc-32233).

Cell Cultures

Immortalized human pulmonary (A549; adenocarcinomic human alveolar basal epithelial cell) and human monocytic (THP1; monocyte isolated from peripheral blood from an acute monocytic leukemia patient) cells obtained from ATCC and gifted from Dr. Aztatzi. A549 are adherent cells while TRP1 cells are suspension cells and floating cells are viable. Both types of cell lines, independently, were grown in the following conditions: Ham F-12 medium with fetal bovine serum (FBS at 10 %), penicillin, streptomycin, neomycin, L-glutamine 200 mM and sodium pyruvate 100 mM (all at 1 %). Cells were maintained at 37 °C in a humidified incubator with 5 % CO2. No morphological changes were observed during maintenance. The exposure to e-liquid bases was performed at 0.5 % FBS to avoid antioxidant protection. A549 cells were trypsinized and cultured at 45,000 cells/cm^2 in culture plates. For all co-culture experiments, THP1 cells were added on top of confluent A459 cells at a 2:1 ratio, on the exposure day. Immediately, the treatments were added and remained exposed for 24 h. Four groups were formed as follows, the control group, the propylene glycol group at 30% with water (PG-30), the vegetal glycerol group at 70 % with water (VG-70), and the group of the mixture of propylene glycol with glycerol (PG-30: VG-70).

MTT Assay

Cell viability was evaluated by MTT (3-(4,5-Dimethylthiazol-2yl)-2,5-diphenyltetrazole Bromide) assay, and it was performed on 96-well plates. Each treatment group of A549, THP1 and co-cultured cells was exposed to 1, 5 and 10% PG-30, VG-70 or PG-30/VG-70 dissolved in exposure medium (0.5 % FBS). After 24 hours of condition exposure, 5 µl of MTT solution (5 mg/mL) was added to each well, and the plate was incubated for 2 h in an ambient incubator (5 % CO2). The medium was subsequently removed and 50 µl of DMSO was added to each well. Plates were placed on a shaker for 1 minute, and 150 µl of deionized water was added. Later, shaken for 1 minute, and the absorbance was read at 590 nm using a reference wavelength of 620 nm in a microplate reader (Labsystem Multiskan NS, Thermo Laboratories, Finland).

Neutral Red Assay

After 24 hours of the exposure condition, the medium was removed, 150 µL of 0.33 % neutral red dye was dissolved in the exposure medium, and then it was added to each well. The plate was incubated for 90-120 minutes at 37 °C in a 5 % CO2 atmosphere. Later, the medium with dye was removed and washed with 150 µL of phosphate-buffered saline (PBS). To solubilize the incorporated dye, 150 µL of a solution containing 50 % ethanol, 49 % MiliQ water, and 1 % acetic acid was added. A shaker was used to facilitate the complete dissolution of the crystals, and the plate was read at 590 nm (Labsystem Multiskan NS, Thermo Laboratories, Finland).

Malondialdehyde (MDA) Assay

Cellular MDA was analyzed by spectrophotometry [22]. Briefly, 185 µL of methyl-2-phenylindole (MPI) and 40 µL of concentrated hydrochloric acid were added to 50 µL of lysed cells in suspension. Then, it was shaken in a vortex and incubated for 40 minutes at 45 ºC. After the incubation, the suspension was centrifuged at 7000xg for 15 minutes. 150 µL of the supernatant was placed in a 96-well plate, and the absorbance was measured using a microplate reader at 590 nm (Labsystem Multiskan NS, Thermo Laboratories, Finland).

Carbonyl Groups Assay

Briefly, 500 µl of DNPH 10 M was added to 100 µl of the sample, mixed, and incubated for 1 h in the dark at room temperature, shaking every 15 minutes. The blank tube has 100 µl of sample and 500 µl of HCl 2.5 M. After incubation, tubes were centrifuged at 1400xg for 10 min, at 25 °C. The supernatant was discarded and 500 µl of trichloroacetic acid (TCA) 2.5 % was added to the pellet. The tube was vortexed and then centrifuged again (1400xg for 10 min, at 25 °C). The pellet was washed with 1 mL of ethanol-ethyl acetate (1:1 v/v) to remove DNPH; the mixture was decanted, and dry for 30 min in an extraction hood. The pellet was resuspended in 500 µl of guanidine 6 M and incubated for 10 min at 37 °C. The absorbance was measured at 370 nm in quartz cells. The molar absorption coefficient of 22 000/M*cm was used to quantify the concentration of protein carbonyl groups based on Fields and Dixon [23].

Lipid Droplets

After exposure, the medium was removed and washed twice with PBS. Then, carefully added 100 µl of formaldehyde 5 % and incubated for 20 minutes. Formaldehyde was removed and washed twice with distilled water (dH_2O). A 100 µl of 60 % isopropanol was added and incubated for 5 minutes. The isopropanol was removed, and 100 µl of the working oily red solution was added and left under slow stirring for 20 minutes at room temperature. Oil red solution (3:2 proportion of oil red and distilled water; dH_2O) was prepared 15 min before use. Finally, the oil red solution was removed and washed with dH2O. Wells images were examined under an inverted digital microscope (EVOS FL Invitrogen-AMF4300, Thermo Scientific57; 10X-lens). Positively red-colored cytoplasm was measured with Image J 1.47 v (National Institutes of Health, USA). Data are presented as an average of four fields. This assay was only performed in A549 cells which are adherent, in contrast to THP1 cells.

Transepithelial Electrical Resistance (TEER)

A549 cells were cultured at a density of 45,000 cells/cm2 in a transwell containing polycarbonate membranes with a 0.4 µm pore and 6.5 mm diameter. TEER was measured with an electrode voltmeter (EVOM; Millipore) before seeding the A549 cells until a confluent monolayer was obtained (TEER no change). THP1 cells were added in a 2:1 proportion to form the co-culture and then were exposed to the vaping liquid base as previously described. Control represents the untreated co-culture condition and PG-30, VG-70 and PG-30:VG-70 the co-culture exposed to these conditions. One of the electrodes was placed in the apical compartment, and the other in the basolateral compartment. TEER was recorded at 0, 1, 2, 4, 6, 8, 12, and 24 h post-exposure with a Milicell ERS-Resistance system (Mers Millipore, Bedford, MA) and expressed in ohms*cm.

Western Blot

Cell cultures were homogenized in lysis buffer (50 mM potassium phosphates, 1 mM DTT (dithiothreitol), 1 mM EDTA (ethylenediaminetetraacetic acid), and 0.1 mM PMFS (phenylmethylsulfonyl fluoride), pH 7.4). The lysate was centrifuged at 7000xg for 10 min, 4 °C. The supernatant was recovered, and the Lowry method was used to quantify the protein. Immunoblot gels were loaded with 30 µg of protein per sample and 2.5 µl of the molecular weight marker (Precision Plus Protein™ WesternC™ Blotting Standards, Bio-Rad or Trident Prestained Protein Ladder, GeneTex) and resolved in 15 % SDS-PAGE gels. Proteins were separated for 2 h at 97 V and then transferred under humid conditions to PVDF membranes (inmobilon® de polyvinylidene fluoride; Merck Millipore) previously activated for 15 min with ethanol; using transfer buffer for 60 min at 90 V. Membranes were blocked with 5 % nonfat dry milk for 1 h at room temperature and later were incubated with primary antibodies overnight at 4°C. The next day, membranes were washed, incubated for 1h with the secondary antibodies, and rewashed at the end. GADPH was used as a loading control (1:1000). Immunoblots were revealed using Immobilon® Forte Western HRP Substrate (Millipore, Burlington, MA, USA) and chemiluminescence was detected in an EC3 Imaging System Chemi HR 410 (Bioimaging System UVP). Protein band density was quantified by the Image J program.

Statistical Analysis

Values are expressed as means ± standard error of the mean (SEM). Statistical analysis was performed using GraphPad Prism® software version 8.1 (GraphPad Software, San Diego, CA). Data with normal distribution (Shapiro-Wik normality test) were compared with One-way ANOVA and Dunnett's or Tukey's post-hoc tests to compare with the control group or between different groups, respectively. P values ≤ 0.05 were considered statistically significant. Three independent experiments were performed in triplicate.

Results

Viability of A549 Cells and Co-Culture with THP1 Cells by Exposure to the Bases of E-Liquids

Cell viability on the individual type of cells and co-culture was performed by MTT and neutral red (NR) assays (Figure 1). In general, MTT presented a decreased viability in contrast with the NR assay, which showed a tendency to increase the accumulation of NR after treatment in treated cells compared to control cells (Figure 1B,1D and 1F). PG-30:VG-70 mixture showed a major reduction in cell viability than those seen in PG-30 and VG-70 individual treatments, and A549-THP1 co-cultured and THP1 cells were more sensible than A549 (seen as a further reduction in viability). Specifically, in the MTT assay after treatment, A549 culture indicates at least an 80 % reduction in cell viability at the concentration of 10% of VG-70, and 5 and 10% of PG-30:VG-70 (Figure 1A). THP1 viability decreases by 80 % at 10 % of PG-30, while PG-30:VG-70 induces changes compared with the control which does not compromise the viability cells (Figure 1C). On the other hand, in the co-culture, the cell´s viability decreased in a concentration-dependent way in VG-70 and PG-30:VG-70 (Figure 1E). Based on these MTT assay results, 1% of e-liquid bases were chosen to perform in the following experiments.

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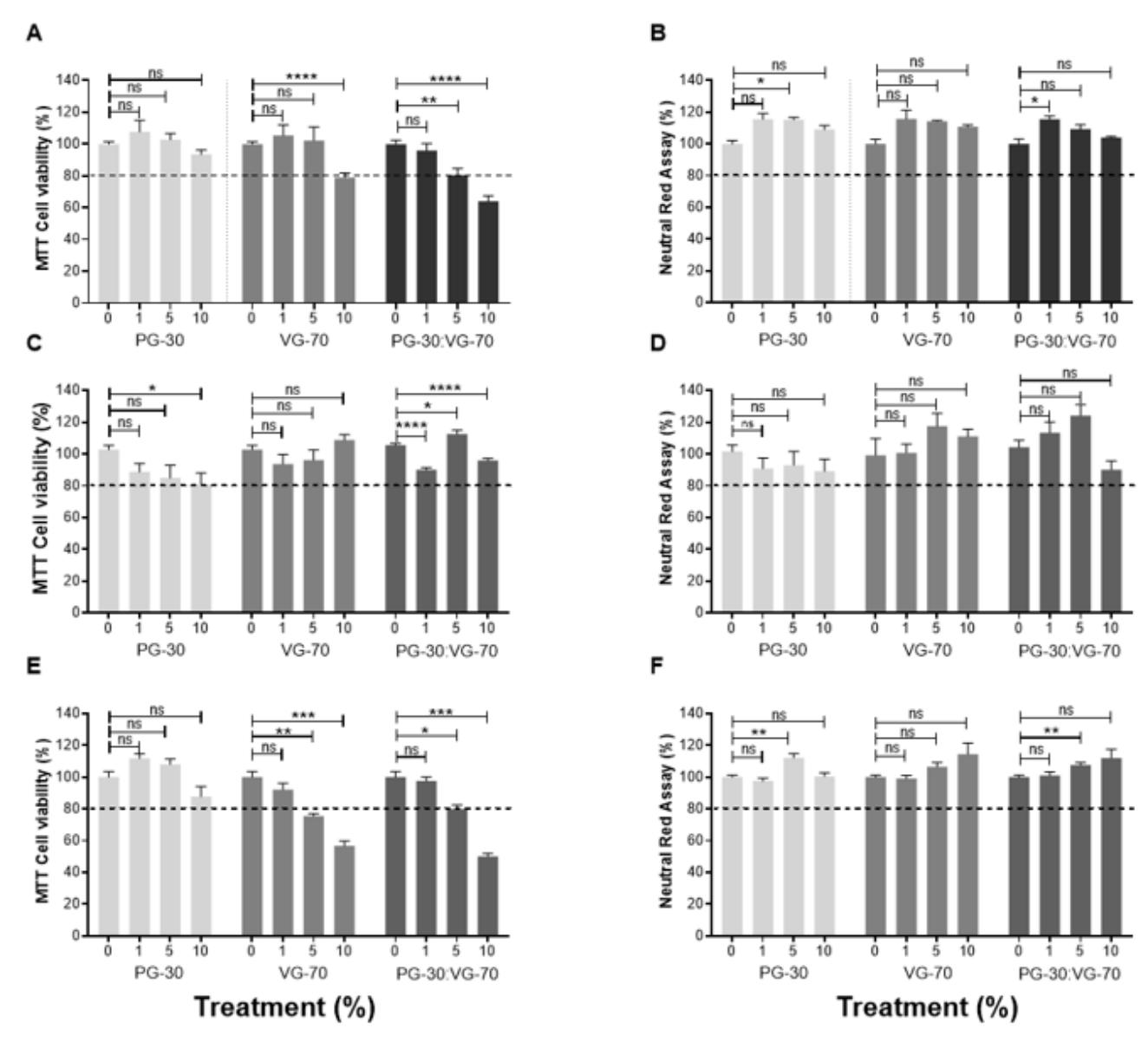

Figure 1: The effect of e-liquid bases on A549 cells, THP1 cells, and co-culture (A549 and THP1 cell) viability percentage. Cell viability was measured by MTT (3-(4,5-dimethylthiazole-2-yl)-2,5-diphenyltetrazole bromide) and neutral red (NR) assays at 24 hours' post-exposure to vaping liquids bases (1-10 %).

A) A549 viability cells evaluated by MTT assay.

B) A549 viability cells assessed by NR assay.

C) Viability on THP1 cells measured by MTT assay.

D) Viability on THP1 cells evaluated by NR assay.

E) Viability on co-culture cells assessed by MTT assay.

F) Viability on co-culture cells by NR assay.

Data showed higher cytotoxicity in the group treated with the PG-30:VG-70 mixture than when they were exposed to the substances separately on all types of cells, this was more evident in co-culture cells than in the individual culture cells. Values are expressed as means ± SEM from three independent experiments by triplicate. One-way ANOVA and Dunnett's post-hoc test. Significance is reported as ns, non-significant difference; *p < 0.05; **p < 0.01; ***p < 0.001; ****p < 0.0001.

Effect of E-Liquid Bases Exposure to Lipid and Protein Oxidation

The lipid oxidation was quantified by malondialdehyde (MDA), one of the final products, and showed a statistically significant increase in the VG-70 and PG-30:VG-70 mixture in co-culture cells (Figure 2A). These changes suggest that PG and VG alter the membrane integrity in the lung. Protein oxidation was assessed by quantification of carbonyl groups, a consistent indicator of protein damage by oxidative stress. We observed an increment in the generation of carbonyl groups by e-liquid bases in all conditions compared to the control group in co-culture cells (Figure 2B). Lipid droplet formation, as a response to cellular stress, was measured by oil red staining in A549 cells after exposure, inducing an additive response to increment its formation in the presence of PG-30:VG-70 mixture in comparison to PG-30 and VG-70 independently, and the control group (Figure 2C,D). Lipid droplet accumulation suggests a similar membrane integrity disruption through lipid oxidation (see discussion).

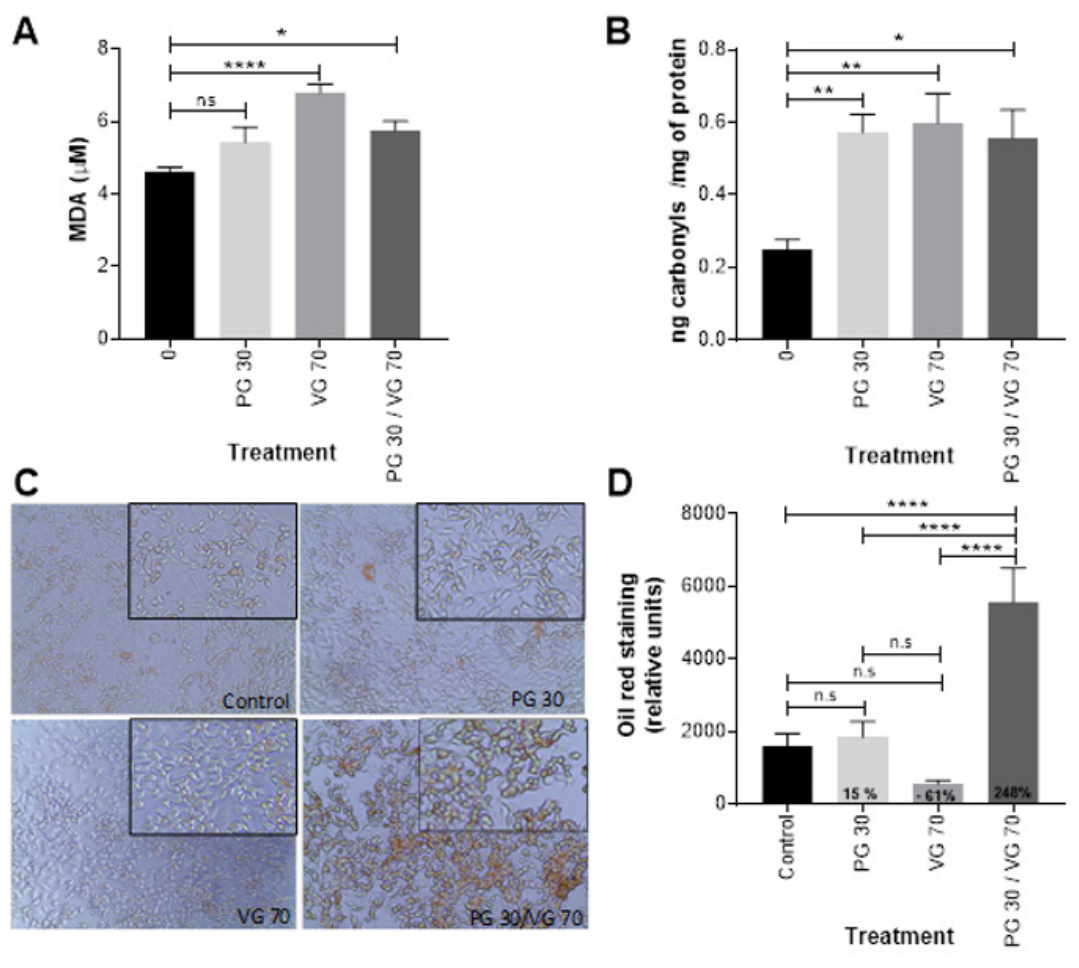

Figure 2: Effect of e-liquid bases exposure to lipid and protein oxidation.

A) Effect of lipid peroxidation, measured by the quantification of malondialdehyde (MDA), after exposure to e-liquid bases on co-culture cells.

B) Effect on protein oxidation, measured by carbonyl groups, after the exposure to e-liquid bases on co culture cells.

C) Effect of e-liquid bases exposure to lipid droplet formation in A549 cells. Representative image (40X) of A549 cells exposed to 1 % of e-liquid bases.

D) Quantitative and statistical analysis of lipid droplets with the ImageJ program.

Lipid droplet formation increased in the presence of the e-liquid bases mixture. Values are expressed as means ± SEM from three independent experiments by triplicate (A and B) or quadruplicate (C) after 24 h of exposure. One-way. ANOVA and Dunnett's (A and B) or Turkey's post-hoc tests.(D) Significance is reported as ns, non-significant difference; *p < 0.05; **p < 0.01; ****p < 0.0001.Oxidative stress induces cellular changes that could include harm to TJ proteins. These proteins keep the integrity of lung epithelia. For this reason, transepithelial electrical resistance (TEER) was measured as a rough indicator of TJ leakiness and measurement of the ion permeability.

Transepithelial Electrical Resistance Alteration in Co-Culture Exposed to E-Liquid Bases

To evaluate the integrity and permeability of the monolayer, TEER was evaluated at 1, 2, 4, 6, 8, 12, and 24 h in A549 and THP1 co-culture cells. TEER decreased after exposure to e-liquid bases; these changes were evident since the first hour and remained so until 24 h post-exposure in all treatments, compared to the control group. PG-30 and VG-70 showed a similar decrement response, but the PG-30:VG-70 had a greater TEER reduction than independent treatments (Figure 3A,B). The area under the curve (AUC) showed a fall of 36.7 %, 33.6 %, and 62.7 % in PG-30, VG-70, and mix conditions, respectively. Data suggest an increased epithelium permeability in all treatments (see discussion). As TJ proteins are considered the rate-limiting factor to paracellular resistance, their protein level change was evaluated by Western Blot.

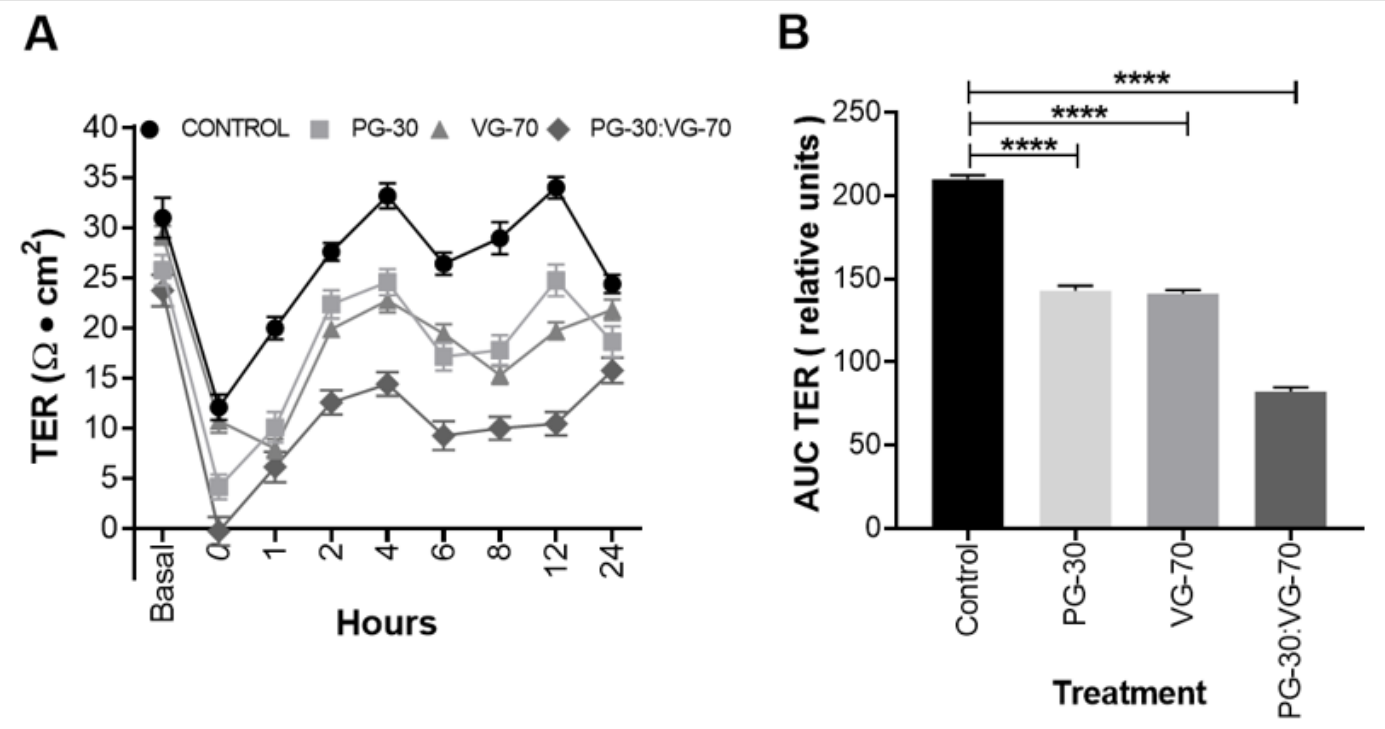

Figure 3: Effect of e-liquid bases exposure on transepithelial electrical resistance (TEER) on co-culture cells.

A) Measurement of TEER over time, in co-culture of cells exposed to e-liquid bases. TEER was evaluated across A549 monolayer and THP1 cells on the upper transwell.

B) Quantitative and statistical analysis of AUC using GraphPad Prism 8.1 software.

Exposure to e-liquid bases decreased TEER on co-culture cells. Values are expressed as means ± SEM from three independent experiments in triplicate. One-way ANOVA and Turkey's post-hoc test. Significance is reported as ****$p < 0.0001$.

Tight Junction Proteins

TJ protein alteration, related to TEER measurement, was detected by exposure to e-liquid bases. The increase in claudin-1 levels after exposure to e-liquid bases was a statistically significant difference noticed in VG-70 and PG-30:VG-70 mix (Figure 4A). Similar results were observed on claudin-4 and occludin (Figures 4C,D). Conversely, claudin-2 levels decreased significantly after all treatments (Figure 4B).

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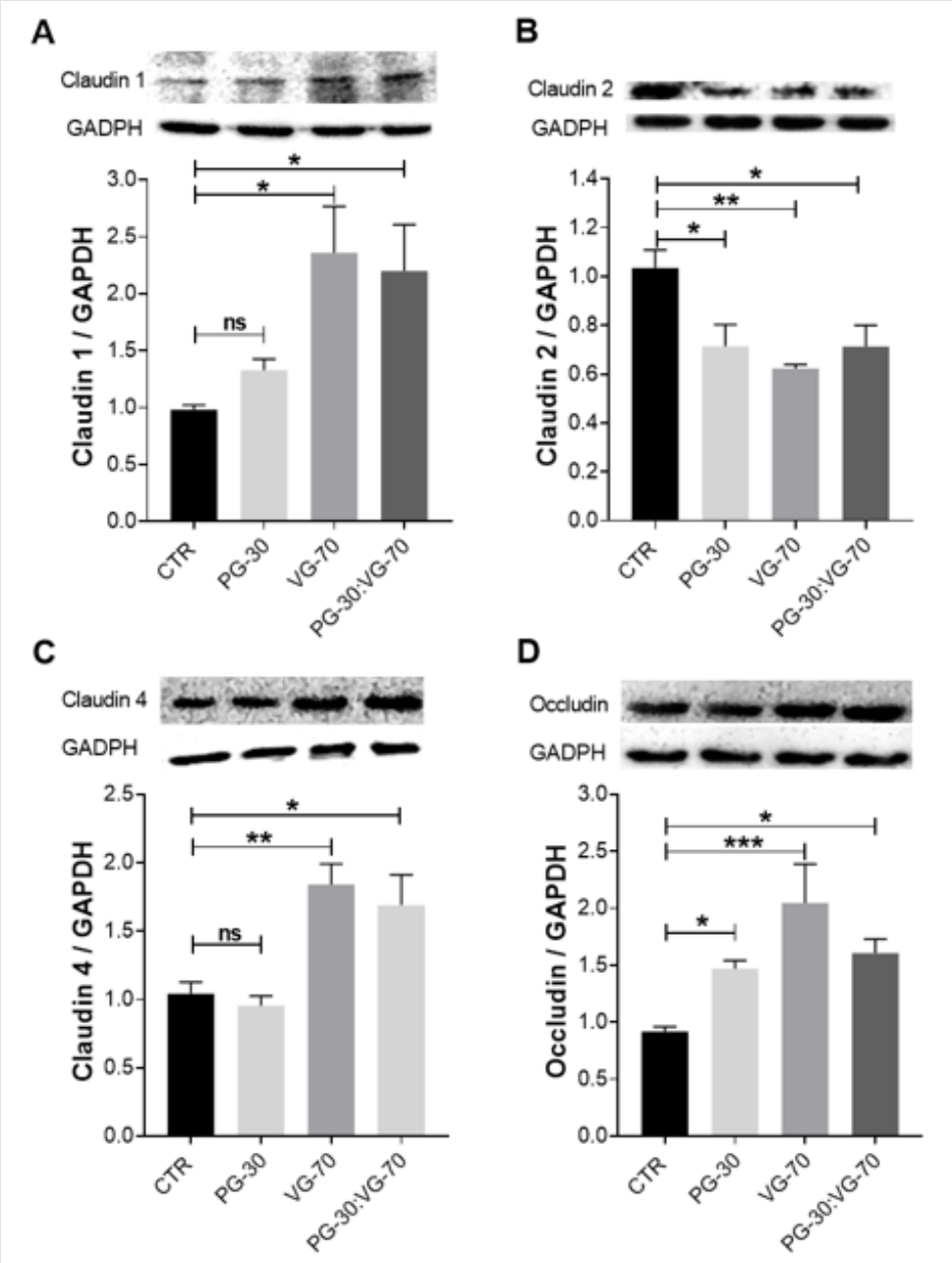

Figure 4: Effect of e-liquid bases exposure on tight junction proteins from co-culture cells. Representative image of Western blot and protein levels of

A) claudin 1,

B) claudin 2,

C) claudin 4, and

D) occludin.

Values are expressed as means ± SEM from four independent experiments in triplicate. Data were compared using one-way ANOVA and Dunnett's post-hot tests. P values ≤ 0.05 were considered statistically significant. Significance is reported as ns, non-significant difference; *p < 0.05; **p<0.01; ***p<0.001.

Discussion

Commercialization and demand for e-cigs caused the innovation and development of these devices with the subsequent variety of flavors. However, both actions share a liquid base that facilitates the availability of the active substance. The use without assessing their potential toxicological effects on the lung epithelium implies a health risk. Handmade e-liquids use higher concentrations of VG to give a better user experience [24]. For this reason, our experimental model was raised with PG-30:VG-70. Few reports have been conducted on the effects of bases or e-liquids without heat [25]. This work evaluated whether bases from e-liquids have the potential toxicity to alter the lung epithelial barrier in a co-culture cells model and their relationship with the induction of oxidative distress due to the exposure to these substances occurring before and after the e-cig use (first- and second-hand inhalation). We found that after 24 h exposure to e-liquid bases at a concentration as low as 1 % induces oxidation of lipids and proteins that contribute to the loss of integrity of the alveolar epithelial monolayer affecting TJ proteins. Collected evidence adds to the fact that although the GRAS classification of vaping liquids recognizes them as safe, it is for aerosolized material, mainly if heated. More tests must be carried out to guarantee consumer safety.

Viability of Cell Culture

The e-liquid bases induced lower viability in a concentration-dependent manner, as evidenced by the MTT assay, being significant for glycerol in the A549 cell line and co-culture cells and propylene glycol in THP1 monocyte cells. It is essential to highlight that this effect was higher in PG-30:VG-70) exposure in all cell cultures. Thus, regardless of the contained additives in e-liquids, exposure to vaping liquid bases in concentrations as low as 5 %, reduced cell viability. A distinctive contrast was observed in the NR assay, a weak cationic dye that preferentially accumulates intracellularly in lysosomes in viable cells. Differences in the viability of treated cells have been attributed to the agent tested and the cytotoxicity used. Depending on the mechanism by which a xenobiotic is expected to act, viability methods may vary. For example, for compounds that act by inhibiting enzymes, enzyme activity assays such as MTT should be used with caution. NR is based on dye uptake and subsequent accumulation in vacuoles or lysosomes; if a compound induces cell surface or lysosomal membrane changes, it will result in low or high NR uptake [26-28]. We hypothesize that e-liquid bases do not significantly affect lysosomal or vacuolar accumulation activity, which does not necessarily affect viability. Increases in NR have been reported for other oily substances such as chloroquine. Therefore, viability is affected by another mechanism, such as enzymatic involvement. E-liquid bases treatment showed an increase in their lysosomal presence that could be related to enhanced lipid droplet formation after exposure to vape bases; however, we measured this in the A549 culture (see below). Thus, this consequence also suggests that vape bases at a concentration performed here are insufficient to induce toxic alterations of lysosomes. Likewise, other authors related this NR accumulation enhancement to lysosomal degradation as a mechanism of protection (lipophagy), which could help to eliminate the disproportionately increased intracellular fatty acids [29]. Furthermore, our data indicate that viability is associated with cell culture type. In this co-culture, which is more affected than individual cultures, the effect is related to the A549 cells' influence. So, in a natural physiological condition, where all different types of cells are in contact, observed effects in the co-culture cells are the best reality representation.

Oxidative Distress Effects by E-Liquid Bases Exposure

The damage to lipids and proteins evidenced oxidative distress. MDA measured a deleterious effect of lipids in the samples known as lipoperoxidation, which showed an increase in all treatments. The specific mechanism by which PG or VG induces oxidative distress or lipoperoxidation is unclear. Fat nature makes it easy to cross the membrane and participate in the metabolic regulation of lipids. Although we did not measure cytokine formation in our work, the induction of stress-related molecules has been reported for other cell lines exposed to e-liquids and their aerosols and is a general mechanism for exposure to xenobiotics. Authors found NADPH induction, Interleukin-1α (IL1-α), and matrix metallopeptidase 9 (MMP-9) secretion on isolated rat heart perfused and cultured human keratinocytes and MatTek EpiDerm™, respectively, which are related to distress [18,30]. Since this is the basis of e-liquids, it is reasonable to assume that such molecule generation can also occur in our model. To prevent mitochondrial lipotoxic damage, recent evidence suggests that lipid droplets are inducible domains that sequester fatty acids to prevent free fatty acids from acting as detergents that disrupt membrane integrity or become cytotoxic at high levels [29-31]. This effect was observed in exposure to the PG-30:VG-70 mix (Figure 2C, D), where lipid droplets increased by 2.48 compared to the control. However, we do not discard that this is a feedback mechanism, and the oxidative distress favors lipid droplet formation. This hypothesis deserves further investigation due to the lack of information about lipid droplet function.

Recent findings in vaping pen users include respiratory illness and a common finding of foamy lipid-laden alveolar macrophages (LLM), which are characterized by the accumulation of fine intracytoplasmic vacuoles into foamy (lipid-laden) macrophages within alveolar spaces. Biopsy from patients with lung injury by vaping showed a morphological similarity to endogenous lipoid pneumonia rather than exogenous lipoid pneumonia [32-34]. These authors hypothesize the lipids into macrophages come from phagocytosed cells during clearance from the lung. Our work shows that alveolar epithelial cells aggregate lipid droplets, similar to the lipid-laden macrophages observed in some cases of vaping respiratory disease and, importantly, without E-vitamin nor tetrahydrocannabinol [35,36], leading to the hypothesis that bases from e-liquids could also contribute to this finding.Carbonylation measures protein damage due to oxidative distress [37]. This study showed an increased carbonyl group formation

in all e-liquid bases a treatment, suggesting that maximum harm by oxidative distress was induced since independent condition. Lerner, et al. [25] measured oxidative distress by oxidizing 2,7 dichlorofluorescein diacetate after exposure to PG and VG unheated [25]. They described an additive amount of oxidants/reactive oxygen species after PG:VG at 1:1 proportion exposure and low levels in individual treatments. They suggested that oxidative distress is responsible for the lipids- and proteins-specific outcomes. Their assay had a different proportion of PG-30:VG-70 mix compared with our work. Other group does not find induction of reactive oxygen species (ROS) after PG/VG (55:45) exposure [17].

Transepithelial Electrical Resistance Effect

TEER assesses the epithelial permeably degree by the ratio of transcellular resistance versus paracellular resistance. TEER values in ohms are indirectly related to permeability but directly evidence the integrity of the monolayer. The leakier the epithelium becomes, as larger this ratio is and visualized by lower TEER units [38,39]. Understanding that alveoli have thin walls to facilitate oxygen and carbon dioxide diffusion, A549 TEER measurements are low. The low TEER values are like those reported by other groups [35,40-42], but they are still known to be capable of expressing TJ proteins such as ZO-1, occludin, E-cadherin, and β-catenin [43]. Downward shifts in TEER were observed from the first hour after exposure for all treatments, with a higher effect observed for the PG:VG mixture treatment compared to the control. The results of the PG-30:VG-70 blend suggest a synergistic effect between the base components and lower TEER measurements, suggesting that the epithelium is more permeable and that TJ proteins are involved, as the effect is shown directly on TEER. TJ proteins comprise a variety of proteins that, together, regulate the paracellular transport of molecules and water. We emphasize occludin and some claudin family members.

Tight Junction Proteins

We found differential changes in TJ proteins after 24 h of exposure to 1% of e-cigs bases. Claudin-1, claudin-4, and occludin augmented their levels, whereas claudin-2 level was reduced after such exposure. Claudins are the major structural components of TJ proteins. They exhibit at least one of the three types of selectivity: cations, anions, or water. Claudin-1 acts predominantly as a barrier claudin (decreases cation permeability) and increases TEER. Knockout mice of claudin-1 died of dehydration in the neonatal period. Claudin-2 acts as a cation-permeable paracellular pore with high conductance to reduce TEER and optimize the water content of lung lining fluid (20,44] Claudin-4 has a controversial behavior, hence depending on the cell type explored. Claudin-4 expression is consistent with alveolar fluid clearance and a protector role for lung acute injury, without altering aqueous fluorescent tracers' permeability in alveolar epithelial cells [45,46]. According to occludin-deficient mice, occludin is not required for the formation of functional tight junctions; but plays an essential role as a proapoptotic signal when junctions are disrupted and increases TEER [46]. Nevertheless, claudin overexpression increased TEER and, at high expression levels, also increased paracellular permeability [47]. Data from e-liquid bases exposure suggest a possible compensatory response where claudin-1, -4, and occludin are upregulated while claudin-2 is downregulated to compensate for the decrease in TEER and, thus, barrier function. Additionally, it suggests a high-water regulation, since claudin-2 allows water entry and reduces its levels, whereas claudin-1, -4, and occludin increase them. The compensation mechanism of the TJ pattern is not sufficient to increase TEER. Hence, restoration of TJ tightness is absent and could be evident as a lung symptom disease. Coordinated activity of TJ protein arrangement expressed in a particular epithelial cell is required to correlate their effects positively. We know several different proteins express simultaneously in the epithelial cell and do not discard those other TJ and claudin proteins that contribute to the final effect. Afterward, cells from the lung epithelium express at least fourteen different claudin mRNAs and proteins [44,46,48]. We consider only a part of the TJ to be a limitation of our study.

Pathophysiological Implication

Heated vaporized e-liquids during vaping maintain their initial components, such as PG and VG, in the same proportion (PG 70:VG 30; [9]). Therefore, knowing the effects of bases and e-liquids alone is essential since they will be vaped along with those produced by thermolysis. Published works focus on e-liquid aerosols, but they usually observe that the effect of e-liquids is similar to that produced by base aerosols [18,44]. These observations imply the relevance of looking at the individual effect to discern the specific causal agent and not only the effect as a whole. Vaping is firstly associated with respiratory diseases such as eosinophilic lung disease, diffuse alveolar hemorrhage, and non-specific acute lung injury [13,49]. The alveolar epithelium and the alveolus must be compromised for any respiratory disease to manifest, which means that the lung cannot compensate for the loss. TJs are critical to the proper functioning function of the alveolus, providing permeability for solutes of different charge, size, and water (exchange and transport across the epithelium [20].

Considering our results on protein carbonylation as a non-reversible modification and the oxidative and inflammatory responses induced by e-cigs reported by others; protein oxidation, cytokines and chemokines production (such as IL-4, IL-6, IL-8, IL-13, IL-1β, IL-17, IL-18, TNF-α and TGF-β) have a high potential to alter TJ expression, damage airway epithelium, recruit neutrophils and lead to respiratory disease [18, 50]. These messenger molecules have been associated with lung edema formation, asthma, respiratory failure, and chronic obstructive pulmonary disease [50,51]. Improving the TJ barrier to allow fluid balance, productive gas exchange, and alveolar fluid clearance would benefit the injured lung and disease symptoms. Lin and collaborators showed in cell and mouse models, using proteins that regulate ions transport (Na+, K+-ATPase, and epithelial sodium channel); a restoration of epithelial/endothelial barrier function and en-

hances expression of the TJs: protein zonula occludens-1 (ZO-1) and occludin after lipopolysaccharide lung injury by reduction of pre-existing permeability increase [52]. Our results indicate that the increase in claudin-1, -4, and occludin were insufficient to restore TEER and this would not be enough to compensate for possible lung dysfunction or damage. However, the mentioned effect after e-cigarette exposure or use has not been reported and should be investigated as a possible alternative to moderate damage to TJ proteins [53,54].

Study Limitations

Some limitations of the work that can be considered as perspectives of the work are studying other tight junction proteins and their localization, observing morphological/physiological changes in the cells, measuring cytokines produced after exposure, performing co-cultures with alveolar macrophages, exposing the cells to aerosols of the e-liquid or the generated condensate and evaluating different PG/VG ratios as the variability in the market is still wide.

Conclusion

Base e-liquid exposure, PG and VG, induces an oxidative distress state that triggers proteins and lipids oxidation, formation of lipid droplets, and disruption of TJ protein homeostasis on A549 and THP1 co-culture cells. PG-30:VG-70 mix and individual treatments had differential effects, depending on assays; while the PG-30:VG-70 mix had synergistic effects (addition) on TEER and lipid droplet accumulation assays, the mix had a similar impact on lipid and protein oxidation, TJ alteration, and viability. Further research is required to know the loss of compartmentalization compromise created by the epithelia.

Conflicts of Interest

The authors declare no conflicts of interest.

Data availability statement

Data are available in https://figshare.com/s/648b5880f-16c14333f6e

Author Contributions

A.L.R., M.P.S.V., O.G.A.A., Y.D.G. and J.R.P.P designed the study (conceptualization). A.L.R., M.A.A.O., O.G.A.A., and I.C.G. performed the experiments. O.G.A.A., M.A.A.O., Y.D.G. performed data analysis. A.L.R. and M.A.A.O., and Y.D.G., wrote the manuscript. Y.D.G. funded acquisition and resources. All authors revised, edited and approved the manuscript.

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