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Validation of an Air-Liquid Interface Model for Lung Epithelium using PreciseInhale® Exposure Studies

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Dedication

This thesis is dedicated to my family, whose unwavering love, encouragement, and sacrifices have been my greatest source of strength throughout my academic journey. To my parents, who taught me my first words and stood by me in my first steps, guiding me every day until I reached this moment. Your support and belief in me have been my foundation.

To my husband and children, for their endless love, patience, and understanding throughout this journey. Your support has been a constant source of motivation, and I am forever grateful for your presence in my life.

I also dedicate this work to my friends, whose companionship, understanding, and positivity helped me persevere through the challenges of this journey.

Lastly, I dedicate this thesis to all those who have inspired and motivated me to reach this point, particularly to Dr. Gabriela Hädrich, whose guidance, expertise, and belief in me have been invaluable. I have learned so much from her throughout this journey, and her support has left a lasting impact on both my academic and personal growth.

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I am most grateful to my family for their constant love, belief in me, and unwavering support throughout this entire experience. Their encouragement, patience, and sacrifices have been my foundation, and I could not have achieved this without them.

I would also like to extend my heartfelt thanks to my friends for their support and camaraderie during this challenging yet rewarding process. Their understanding, positivity, and motivation were a great source of strength, and I am fortunate to have had them by my side.

Thank you all for making this achievement possible.

Chatha Fares

Abstract

The development of reliable *in vitro* hazard assessment models is crucial for replacing or refining animal testing. Traditional *in vitro* pulmonary cytotoxicity studies often rely on submerged culture conditions, which fail to replicate real-life cell-cell communication and particle interactions. To address these limitations, air-liquid interface (ALI) models, such as Calu-3 cell lines, offer a more realistic approach, mimicking lung epithelial barriers. Advanced exposure systems like the PreciseInhale® integrated with the XposeALI® module enable controlled aerosol delivery to ALI-cultured cells, allowing precise and reproducible inhalation toxicology studies being capable of exposing 3D-cultured bronchial epithelial cells to aerosolized substances, including nanoparticles and pollutants, while ensuring uniform particle deposition. Despite its advantages, such as minimal aerosol contamination and reduced exposure times, limitations include low throughput and a time-intensive cleaning process. This study aimed to evaluate the health status of ALI-cultured Calu-3 cells exposed to clean air using the XposeALI® system, providing critical insights into their suitability for robust and reliable inhalation toxicity assessments. The exposure had no cytotoxic effect on membrane integrity and mitochondrial activity, as well as no disruption in the cellular tight junctions when accessed via microscopy and TEER measurements. The clean air exposure also did not cause a pronounced inflammation for the parameters tested (IL-6, IL-8 and TNF-alfa). Further validation is needed for the inflammation control using LPS. The ALI exposure model was implemented successfully and it is ready to be used for research in drug development and environmental toxicology.

Zusammenfassung

Die Entwicklung zuverlässiger in vitro-Modelle zur Gefahrenbewertung ist entscheidend, um Tierversuche zu ersetzen oder zu verbessern. Traditionelle in vitro-Studien zur pulmonalen Zytotoxizität basieren häufig auf submersen Kulturbedingungen, die die Zell-Zell-Kommunikation und Partikelinteraktionen im realen Leben nicht nachbilden können. Um diese Einschränkungen zu überwinden, bieten Air-Liquid-Interface (ALI)-Modelle, wie die Calu-3-Zelllinien, einen realistischeren Ansatz, der die epitheliale Barriere der Lunge nachahmt. Fortschrittliche Expositionssysteme wie das PreciseInhale® in Kombination mit dem XposeALI®-Modul ermöglichen eine kontrollierte Aerosolabgabe an ALI-kultivierte Zellen, was präzise und reproduzierbare Inhalationstoxikologiestudien erlaubt. Diese Systeme sind in der Lage, 3D-kultivierte bronchiale Epithelzellen Aerosolen, einschließlich Nanopartikeln und Schadstoffen, auszusetzen und dabei eine gleichmäßige Partikelablagerung sicherzustellen. Trotz ihrer Vorteile, wie minimaler Aerosolkontamination und verkürzter Expositionszeiten, umfassen die Einschränkungen eine geringe Durchsatzrate und einen zeitintensiven Reinigungsprozess. Ziel dieser Studie war es, den Gesundheitszustand von ALI-kultivierten Calu-3-Zellen nach einer Exposition mit sauberer Luft unter Verwendung des XposeALI®-Systems zu bewerten, um kritische Einblicke in deren Eignung für robuste und zuverlässige Inhalationstoxizitätsbewertungen zu gewinnen. Die Exposition hatte keinen zytotoxischen Effekt auf die Membranintegrität und die mitochondriale Aktivität sowie keine Störung der Zell-Zell-Kontakte, was mittels Mikroskopie und TEER-Messungen beurteilt wurde. Die Exposition mit sauberer Luft führte zudem zu keiner ausgeprägten Entzündungsreaktion bei den getesteten Parametern (IL-6, IL-8 und TNF-alpha). Für die Validierung der Entzündungskontrolle mit LPS sind weitere Untersuchungen erforderlich. Das ALI-Expositionsmodell wurde erfolgreich implementiert und ist bereit für die Anwendung in der Arzneimittelentwicklung und Umwelttoxikologie.

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1. Introduction

Establishing reliable and robust *in vitro* hazard assessment models is an important strategy to replace or refine animal testing. However, *in vitro* methodologies require the evaluation of the model regarding its transferability and reproducibility. An ideal model would establish a good correlation between in vitro and in vivo data, shorten the drug development phase, and improve product quality (1). Most *in vitro* studies to assess pulmonary cytotoxicity have been performed using lung cells (cell lines and primary cells) cultured under submerged conditions with the tested compounds (drugs, pollutants, etc.) added directly into the cell culture media. However, such experimental setups do not reflect realistic cell-cell communication and cell-particle interactions in real-life scenarios (2). Moreover, when exposing epithelial cells *in vitro* to particles under submerged conditions, a substantial fraction of the particles will either remain in the liquid or be lost to the lateral walls of the culture vessel, which alters the dose of particles as well as the interaction with the cells.

As an *in vitro* lung cell model, the Calu-3 cell line, derived from human bronchial adenocarcinoma, has been used extensively in lung barrier models (3–6). These cells are regarded as a good model for lung airway epithelia since they can form a pseudostratified cell layer with a proper barrier function at the air-liquid interface (ALI). Moreover, the presence of tight junctions and the secretory activity make the Calu-3 cell line a promising tool for lung drug absorption studies (2). Lung models that can be exposed via the air, through an air-liquid interface (ALI) are promising in vitro models for evaluating the safety of new compounds or pollutants after inhalation exposure (7) and a well-described respiratory model to study the effect of compounds on the human airway epithelial cells (8).

Cells exposed to ALI experimental conditions (Fig. 1) often get more oxygen and shift cellular respiration into a more aerobic state. Consequently, oxidative cellular metabolism is accelerated, which boosts cellular differentiation. Therefore, when the cells are cultured under ALI conditions it could lead to a decrease of biological effect after particle exposure compared with submerged conditions, due to lower oxygen availability that increases a pro-inflammatory response (1). For a better understanding of the possible toxic effect of a substance, it is important to

characterize well the cell health status of the unexposed cells under ALI before accessing the possible effect of substances after exposure.

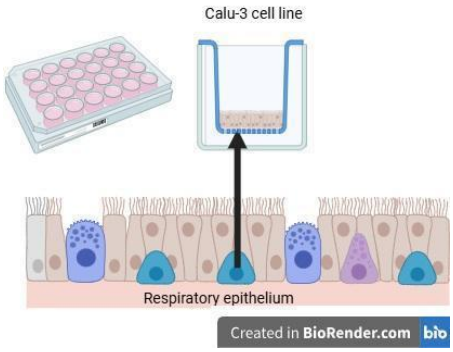

Figure 1. Schematic representation of the CALU-3 cells cultured on inserts under ALI for cell exposure assays. The image was created using Bio Render (https://app.biorender.com).

In the past few years, some in vitro exposure systems were developed to simulate human respiratory exposure. As an example, we can cite the Vitrocell®, CULTEX® and PreciseInhale®. These devices can precisely deliver controlled doses of air-bone substances (particles, aerosols, gases, and nanoparticles) to ALI-cultured cells, enabling accurate, reproducible studies of inhalation toxicology and drug delivery in a way that closely mimics real-world inhalation exposure (9). The PreciseInhale® was recently installed in the Department of Pharmaceutical Technology and Biopharmacy of the University of Vienna and is an advanced inhalation exposure system developed in Sweden by InhalationSciences® to deliver accurately controlled doses of aerosols or particles directly to cell culture (10), animal models (11) or humans (12).

The aerosolization process in the PreciseInhale® system utilizes compressed air at pressures of up to 160 bar to effectively disperse particles by breaking up agglomerates in the powdered material. Even cohesive nanoparticle powders are efficiently dispersed using this method, enabling minimal usage of powdered substances. A standout feature of this system is its advanced dosing mechanism, which includes the Casella light dispersion technology. This approach ensures precise control of the dose delivered to the cell surface, with adjustments made in real-time by a computer during each experiment (13). This technology allows for precise dosing and uniform particle distribution, which are essential for high-quality, reproducible data in respiratory toxicology and pharmaceutical research.

The XposeALI® is a sophisticated *in vitro* exposure module that integrates with the PreciseInhale® platform. This system is notable for its ability to expose 3D cultured bronchial epithelial cells to aerosolized substances, enabling precise and dynamic toxicity assessments of inhaled particles, including nanoparticles and other airborne pollutants (9,10). To the best of our knowledge, a full understanding of the CALU-3 cells exposed to clean air under ALI using the XposeALI® connected to the PreciseInhale® was not accessed before.

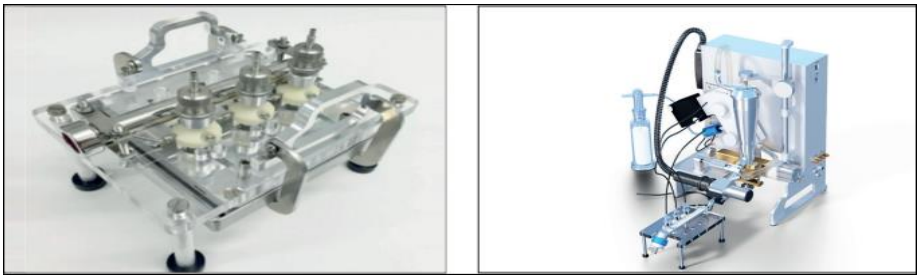

Figure 2. With XposeALI® (right) exposure model and PreciseInhale® (left) (adapted from Inhalation Sciences Sweden AB; https://www.inhalation.se/instruments/XposeALI®-cell-exposures/)

The XposeALI® module produces highly concentrated aerosols, allowing for shorter exposure times. This reduced exposure duration minimizes the time cells spend outside the incubator, a critical factor for the viability of cells. Additionally, efficient aerosol deposition can be achieved in the ALI cell culture models without the need for more complex techniques such as electrostatic deposition. The exposure setup is designed to focus particle deposition exclusively on cell layer surface, avoiding the insert walls. This is facilitated by a reverse airflow pattern that directs excess aerosol toward a central outlet, where it is captured by filters on the exposure hood. This design not only ensures uniform distribution of particles across the cell surface but also reduces the risk of aerosol contamination in the laboratory environment (13).

Besides the aerosol generation precision, XposeALI® enables cell exposure to respirable aerosols using the ALI setup cells exposed to air at the” luminal” side and media at the ”vascular” side, mimicking the conditions in the lung (13), which makes the studies of the cellular effects induced by airborne particles robust and reliable. The XposeALI® system offers the scope for exposing ALI models not only to drugs but also to dry aerosolized environmental pollutants (2). As an example, Ji

et al., 2017 (13) used the XposeALI® for exposure of ALI primary bronchial epithelial cells co-cultured with fibroblasts to dry aerosolized Pd nanoparticles. In this study, they combined the 3D-cell model with controlled aerosol exposures thereby managed to deposit nanoparticles on a differentiated cellular layer cultured at ALI.

While acknowledging the exceptional advantages of the XposeALI®, the device has some limitations, for example, only three inserts are allowed to be exposed at the same time. The “unexposed” group should be exposed in another run to expose the cells to clean air. The cleaning process involves meticulous attention to every detail and, therefore time-consuming. To deepen understanding of the device, this study aimed to evaluate the health status of ALI-cultured CALU-3 cells following exposure to clean air using the XposeALI®. The goal was to determine better when cellular responses are sufficiently robust to be deemed reliable and acceptable.

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2. Material and Methods

2.1. Cell culture

The Calu-3 cells (ATCC-HTB-55; passage number 8–12) were cultured in Dulbecco's Modified Eagle Medium (DMEM)/F-12 (Thermo-Gibco, 31331093) with 10% Fetal Bovine Serum (FBS) (FBS Superior, Merck. Biochrom, S0615), and 1% penicillin/streptomycin (Penicillin/Streptomycin 10000U/ml, (Thermo Fisher-Gibco, 15140122) at 37°C and 5%CO_2; the medium was refreshed every 2nd or 3rd day. The cells were grown until confluency in T75 flasks and split 1:6 once a week.

2.2. Air-liquid interface (ALI) culture

When reaching approximately 80% confluency in the cell culture T75 flask, cells were detached enzymatically (0.25% trypsin-EDTA) and subsequently seeded (seed density: 1.0×10^5 cells/cm^2) on the apical side of transparent inserts (0.4 μm pore membrane, FALCON, USA) fitted in a 12-well plate with 0.5 mL cell suspension on the apical side and 1.5 mL culture medium was added into the basolateral side. The submerged culture period to reach confluence was set at 7 days under standard conditions (5%CO_2 at 37°C). The basolateral medium was changed every second day, and the apical chamber was washed by PBS. After 7 days, the TEER was measured, and the apical medium was removed from the inserts to obtain the ALI conditions. The morphology of the epithelial cell models (Calu-3) in the inserts was monitored over time via an optical microscope (ECHO) with a 20-time objective lens. The following methods were reproduced according to previously published SOPs and will be described briefly in each section (14).

2.3. Transepithelial electrical resistance (TEER) measurement

As an indicator of barrier integrity, transepithelial electrical resistance (TEER) was measured using an Evom3 Voltohmmeter equipped with 4 mm chopstick

electrodes (STX-4) (World Precision Instruments Inc., FL, USA) before and after the exposure to the clean air. Since the cells were kept at ALI, 0,5 mL of medium was added onto the apical side of all the inserts and the plate was incubated at 37°C and five%CO_2 for 30 minutes before the measurement. All TEER values were corrected for the resistance of a cell-free insert (≈130 Ω) and the surface area of a 12-well insert (1,12 cm^2).

2.4. XposeALI® clean air exposure

To mimic the *in vivo* exposure conditions of the lung, the cell monolayer was aerosolized under ALI conditions using the XposeALI® attached to the PreciseInhale® three times for 240 seconds. The aerosol was generated using the high-pressure aerosol generator of the PreciseInhale® exposure platform. In brief, three inserts containing ALI Calu-3 cells were exposed to the clean air airflow and compressed air of 100 bars was used to aerosolize into the 300 ml holding chamber. The clean air aerosol was pulled from the holding chamber with a constant flow rate (120 ml/min) and diverted into triplicate branch exposures at a flow rate of 10 ml/min per branch. Additionally, in the exposure module, the inserts were always in contact with 4 ml of cell culture medium on the basolateral side to avoid any physiological stress to the cells. The experimental timeline is schematic represented in Figure 3.

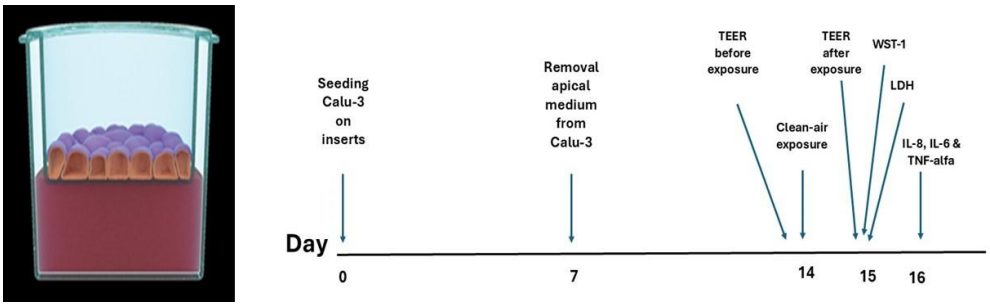

Figure 3. Schematic overview of the experiment workflow for culturing and exposing the Calu-3 ALI cells to the XposeALI® system.

2.5. Cell viability and inflammatory cytokine release

2.5.1. Membrane integrity (Lactate dehydrogenase (LDH) release

The cytotoxicity was accessed using the cell membrane integrity, lactate dehydrogenase (LDH) release in the basolateral medium was measured (Cytotoxicity Detection Kit (LDH), Roche Diagnostics GmbH Mannheim Germany Cat. No. 11 644 793 001). Briefly, 100 µL of supernatant and 100 µL reaction reagent were successively added into a 96-well flat-bottomed plate and incubated in the dark for 20 min at room temperature. After that, the absorbance was measured on the wavelengths 490 nm and 620 nm. All LDH values were corrected for the maximum LDH release when cells were incubated with lysis buffer (2% Triton X-100, Thermo Fisher Scientific Inc., the Netherlands) for 30 min. The lysate was collected for LDH measurement. To determine the percentage of cytotoxicity, calculation with the controls using equation 1, according to the manufacturer's protocol was used.

$$Cytotoxicity\ \% = \frac{exp.value - untreated}{triton - untreated} \times 100$$

Equation 1. Calculation of the cytotoxicity (%) according to the LDH kit.

2.5.2. Mitochondrial metabolic activity

Cell mitochondrial activity was evaluated using the WST-1 cell Proliferation Reagent (No. 11644807001, Roche Diagnostics GmbH, Mannheim, Germany). At 24 h after exposure, and after collection of supernatants for cytokine analysis (see paragraph below), WST-1 reagent (1:10 WST-1 (In DMEM-F12 incomplete)) was added to the apical side of the inserts for (30 min). Subsequently, 100 µl of supernatant of each insert was transferred to a 96-well plate in duplicate. If the absorbance is greater than 0,2 and lower than 2 OD for all the wells, the reading was performed for the other 100 µl. Absorbance was measured using a spectrophotometer (EPOCH 2, BioTek) at a wavelength of 440 nm and a reference wavelength of 620 nm. The equation 2 was used to calculate the cytotoxicity values in percentage.

$$Cytotoxicity\ \% = \frac{(100 \times (Control - Sample))}{Control}$$

Equation 2. Calculation of the cytotoxicity according to the WST-1 kit. The control is the absorbance of the unexposed cells at 440 nm.

2.5.3. (pro)inflammatory cytokines: (IL-8, TNF-alpha, IL-6)

To test the inflammatory response, inflammatory cytokines (interleukin IL-8, IL-6 and tumor necrosis factor (TNF)-α) in the apical medium were quantified using enzyme-linked immunosorbent assay (ELISA) kits (Invitrogen by Themo Fisher Scientific Inc., Austria) otherwise stated, the manufacturer's protocol was followed. For the TNF-α the capture and detection antibodies had to be used in a concentration 1,5x higher than the manual due to low signal in the calibration curve. The samples were added to pre-coated wells 24 hours after the clean-air exposure. Absorbance was measured using a spectrophotometer (EPOCH2, BioTek Instrument, USA.) at a wavelength of 450 nm and a reference wavelength of 570 nm.

3. Resuls

3.1. Cellular morphology

The morphological observation of the cells under microscope 24 hours after the XposeALI® exposure showed the effectiveness of the Triton-X to cause membrane disruption. There were no visible differences between the inserts exposed to the clean airflow when compared to the unexposed cells (Fig. 4).

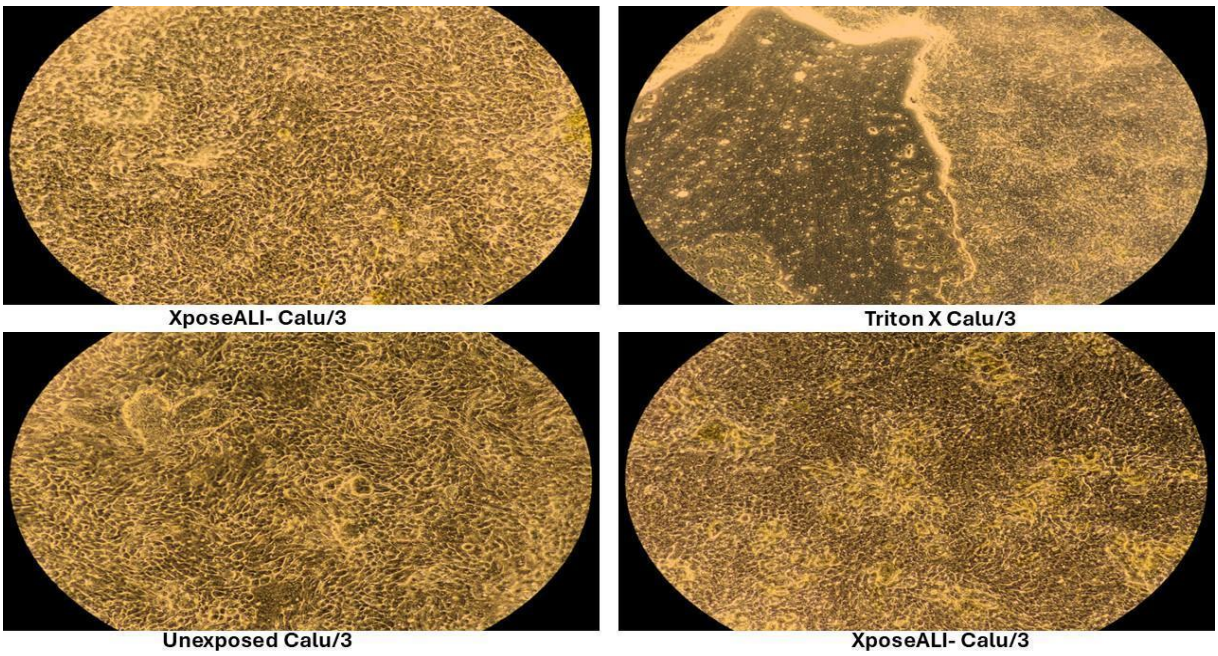

Figure 4. Overview of the morphology of the epithelial cell models (Calu-3) onto the inserts after 24h of the exposure process via an optical microscope (ECHO) with a 20-time objective lens.

3.2. Transepithelial electrical resistance (TEER)

The TEER values were measured before and 24 h after exposure (Fig. 5). The exposure process showed no disruptive effect on the tight junctions what could be seem by the TEER values of the exposed cells compared with the unexposed cells. After 24 h all the cells showed a slight increase in the TEER values, what was expected since the healthy cells would still be replicating. In contrast, the triton affected the monolayer integrity in a significant manner due to the disruptive effect expected from this control. The LPS control also showed no effect on the TEER values.

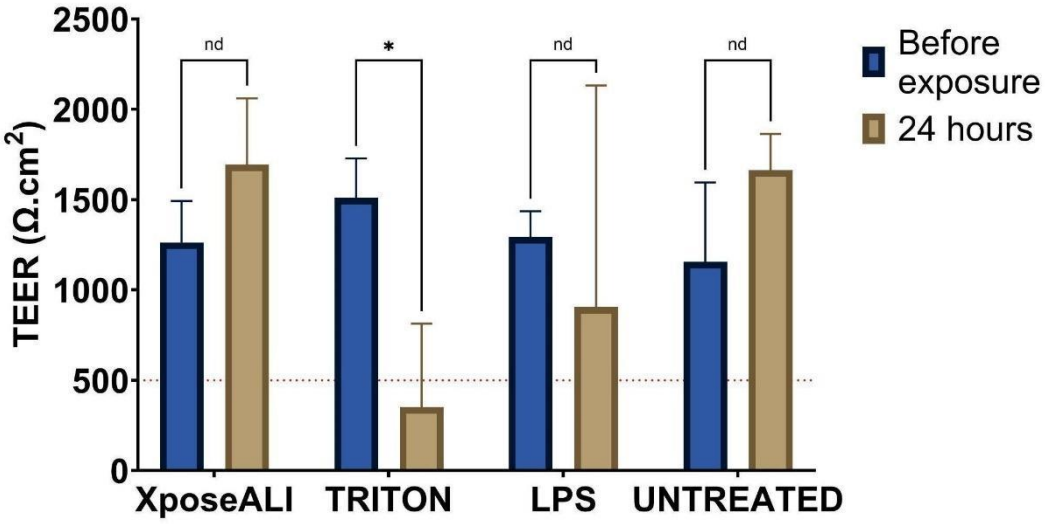

Figure 5. Overview of TEER values of Calu-3 cells after 24 h of XposeALI® exposure. The exposures showed no significant effect on TEER values ($p > 0.05$). The triton control caused a significant reduction in TEER values compared to the unexposed group ($p < 0.05$). Data are presented as mean ± SD from N = 4 experiments.

3.3. Cell viability

There was no significant effect on membrane integrity after 24 hours of the clean air exposure (Fig. 6) process on the LDH release and the cytotoxicity of the exposed cells (~2%) compared with the unexposed cells (>1%). Similarly, the cells exposed to the control LPS showed no increase in cytotoxicity (~2,5%) compared to the

unexposed cells. The triton was used as a positive control of membrane disruption, therefore the most pronounced increase in cytotoxicity was seen this group (<100%). The cytotoxicity percentage higher than 100% is due to the inherent methodological differences such as sensitivity, cell density, or experimental conditions, which can lead to slightly higher-than-expected LDH release (15).

According to the guidelines, a smaller reduction in viability (e.g., 10–20%) could result from experimental noise or non-specific stress, rather than true cytotoxicity. A reduction of 30% or more reliably reflects adverse effects like cell death or metabolic disruption, critical indicators of cytotoxicity (16). Therefore, the cytotoxicity which was measured using cell proliferation reagent WST-1 to evaluate the cytotoxicity (Fig. 6) using the mitochondrial activity showed that there was the irrelevant effect on the mitochondrial metabolic activity after 24 hours of exposure (5,5%) compared with the unexposed cells (0%). Although the cytotoxicity percentage in the LPS-treated cells was numerically higher (23,1%, according to the guidelines, less than 30% of viability reduction is accepted. Triton-treated cells (positive control group) showed the highest cytotoxicity percentage (100%).

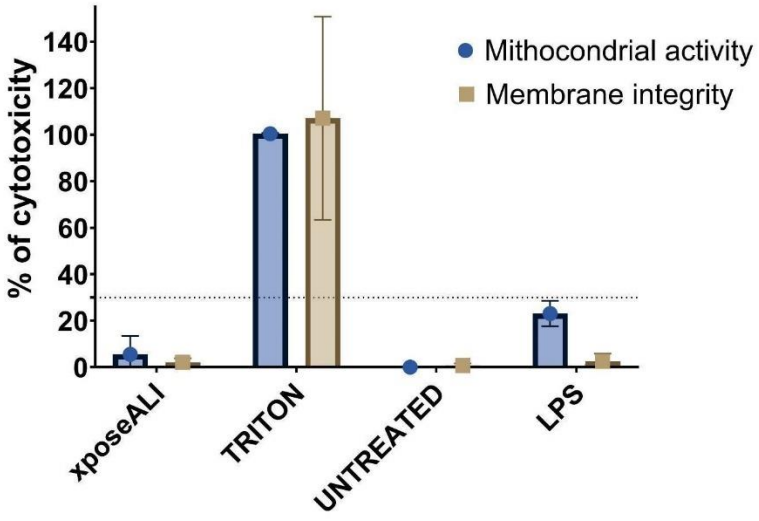

Figure 6 Comparison of cytotoxicity (%) measured using the mitochondrial activity assay (WST-1) and the cell membrane integrity assay (LDH) after 24 hours of ALI exposure using XposeALI®. Data are presented as mean ± SD of 3 independent experiments.

3.4. (pro)inflammatory cytokines: (IL-8, TNF-alpha, IL-6)

The exposure procedure did not increase IL-8 levels, as IL-8 concentrations were comparable across exposed, unexposed, and Triton control groups when measured against the cell culture medium baseline (Figure 7). The LPS control showed a significant decrease in IL-8 levels compared to the unexposed cells.

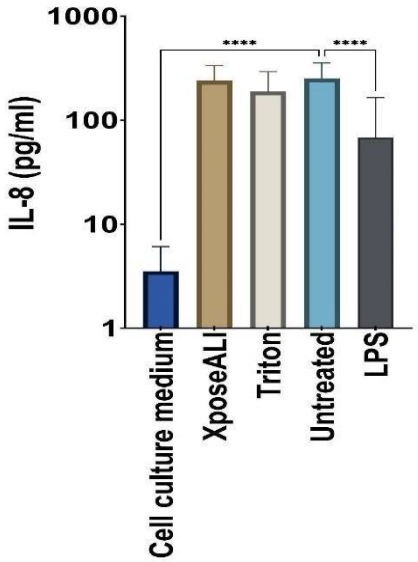

Figure 7. Overview on IL-8 concentrations measured after 24h of ALI exposure using XposeALI® Calu-3 cells. The results are expressed as mean±SD of 3 experiments (3 different passage numbers) and n=3 inserts per experiment.

The cellular exposure to clean air resulted in a 2.8-fold increase in IL-6 concentration compared to unexposed cells, as shown in Figure 8. Among the controls, triton exhibited the most substantial IL-6 elevation, with levels approximately seven times higher than those of unexposed Calu-3 cells. In contrast, no significant increase in IL-6 levels was observed in the LPS control. We would like to emphasize that for future experiments, normalizing interleukin concentrations to cell numbers will be an essential step to ensure accurate and consistent results.

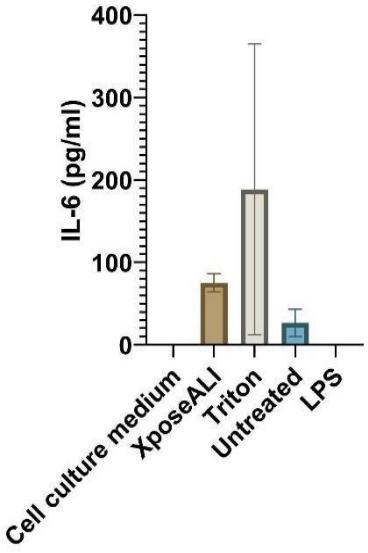

Figure 8. Overview on IL-6 concentrations measured after 24h of ALI exposure using XposeALI®. Calu-3 cells groups: Triton-treated Calu-3 cells (control group), Unexposed cells, exposed cells, LPS treated Calu-3 cells. Data represent mean ± SD

The TNF-α assay conducted on the apical medium of Calu-3 cells consistently showed negligible or "0" levels of TNF-α across all samples. This result was observed even after modifying the ELISA protocol by increasing the concentrations of capture antibody and detection antibody to improve signal detection. Since calibration curves were added to all the plates we could exclude experimental error. The data, including calibration curves, tables, and calculations, are presented as supplementary material. The fact that no TNF-α was quantified would be significant for the next step of this study when a co-culture model with macrophages will be established.

3.5. Acceptance criteria

The acceptance criteria were proposed based on the CV% values, as cell-based experiments are considered reliable when presenting a CV% < 50% following updated trustworthy guidelines (17) for *in vitro* testing, variability below this threshold indicates consistent and reproducible results. Data points exceeding this limit in the current study highlight the need for optimization in experimental protocols to improve reproducibility and ensure data reliability (Figure 9). The CV% values were calculated according to the equation 3.

$$CV\% = \left(\frac{Standard\ Deviation(SD)}{Mean}\right) \times 100$$

Equation 3. Coefficient of variation (%) calculation using the standard deviation and the mean of all experiments made for each treatment group.

The results obtained here indicate that untreated samples demonstrated acceptable variation for the TEER measurements, corroborating with the morphological microscopical analysis (Fig. 9) indicating an intact cell monolayer. However, the treatments with LPS showed high variability after the exposure, potentially indicating disruption of epithelial barrier integrity. For the future, a study testing different concentrations should be performed to stablish a reliable LPS control. Further studies including a higher N of each treatment must be conducted to better understand the inherent variability caused by the exposure and shorten the variation of some parameters. We hypothesized that normalizing the parameters with the cell number would help to make the effects less pronounced.

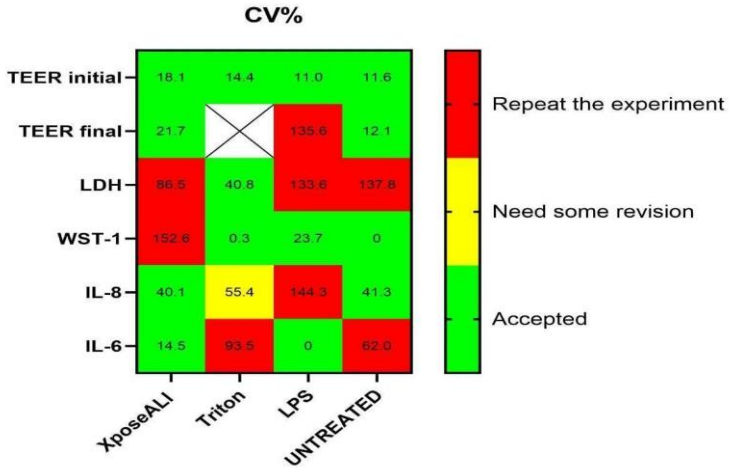

Figure 9 Overview of Coefficient of Variation (CV%) of the Parameters (TEER, Cytotoxicity % based on LDH, Cytotoxicity % based on WST-1, IL-8, IL-6) Calu-3 cells groups: Triton-treated Calu-3 cells (control group), Unexposed cells (untreated), LPS treated Calu-3 cells, Exposed cells. Data represent mean ± SD of 3 different passage numbers and a n=3 for XposeALI and Untreated and n=1 for triton and LPS.

4. Discussion

The most up-to-date guidelines for toxicity studies (18) are limited by interspecies comparison because they are made for murine models. It´s well described that the human respiratory tract anatomy and function are different, plus the particle's aerodynamic sizes and behaviour are significantly different between rodents and humans which could lead to wrong conclusions on human hazard potential (19). In this vein, the work presented here shows the possibility of testing drugs or pollutants in a more human-like manner combining the XposeALI® aerosol capability with the human-derived lung epithelium cell line. Therefore, to ensure that a cell-based model produces reliable data, it´s vital to well understand the baseline characteristics of unexposed cells because this will determine that the observed changes after exposure to drugs or pollutants can be accurately attributed to the tested compounds rather than inherent variability or pre-existing conditions in the cell populations (20). Therefore, characterizing the baseline parameters to know the inherent variability of the model plays a pivotal role in judging when the experiments are acceptable.

The TEER measurements are routinely conducted to evaluate the cell monolayer integrity. Hence, our data showed that absolute values might differ as also observed by Braakhuis, et al., 2023 in the intra-laboratory comparison data set (7). This variability is also extensively described in the literature for TEER values and interlaboratory variations highly likely due to the high sensitivity of TEER to both the exact positioning of the electrodes, device model, cell culture conditions, type of insert and even slight corrosion levels. The TEER may, however, be useful as a control for assay performance within specific and well-known conditions (21). Considering this inter and intra-laboratory variability, the TEER values are applicable for cell culture characterization by each laboratory but not appropriate as a strict absolute comparison tool. However, despite the variations between labs, it´s well agreed that a reduction in TEER values in CALU-3 cells typically indicates a compromise in the integrity of the tight junctions between these cells and that´s why it is vital to validate the TEER measurements before setting up a new method.

The CV% for the TEER values in the unexposed group as < 50% before and after the experiment are an indication of an intact monolayer and it could be confirmed when the inserts were analyzed using microscopy. The variation observed was

consistent with those of the compared study performed by Braakhuis et al., 2023 for exposed and unexposed cells since both studies found no effect on TEER values. However, there were discrepancies in the outcomes between the two studies concerning LPS control (7). In the study by Braakhuis et al. (2023), LPS exposure did not impact TEER values, whereas here we observed a reduction in TEER values of the LPS-treated group. We attributed this effect to two main reasons; first, the LPS control should be tested varying the experimental conditions (concentration and time) with the results normalized by the cell count. The second and most acceptable explanation is the differences in LPS exposure methods of LPS on the apical side of the Calu-3 cells between the two studies being exposure using the VITROCELL® Cloud12 system (14) and direct pipetting used here. For future studies, we will evaluate different concentrations of LPS and normalize the results for the cell number. The main reason we used the LPS is to have a control for the inflammatory response, and the control did not show the expected increase in IL-6 and IL-8 levels, for this purpose the normalization should also provide more robust data.

Cytotoxicity is a crucial endpoint for the final interpretation of toxicological results to differentiate between specific and unspecific effects (7). Here, the cytotoxicity percentage was calculated depending on the two methodologies (LDH assay and WST-1). The LDH assay detects lactate dehydrogenase, an enzyme released into the cell culture medium when the plasma membrane is compromised, which makes LDH an excellent marker of membrane integrity (5), and the WST-1 assay measures mitochondrial dehydrogenase activity as an indicator of cell viability being particularly sensitive for detecting changes in metabolic activity (5). In this study, both methods found no toxic effect of the XposeALI® exposure process itself on the Calu-3 cells. The cytotoxicity percentage using both methods was under 30%, so according to the LDH results the cytotoxicity values are all considered subtoxic according to the OECD guidelines (18). According to the guidelines of the Biological and Experimental Significance, a smaller reduction in viability (e.g., 10–20%) could result from experimental noise or non-specific stress, rather than true cytotoxicity. A reduction of 30% or more reliably reflects adverse effects like cell death or metabolic disruption, critical indicators of cytotoxicity (16).

The mitochondrial activity test WST-1 provided different values of cytotoxicity percentage than the LDH, which can indicate a higher sensitivity of the sub-lethal

toxicity of the WST-1 compared with the LDH test. The WST-1 showed also less variability in the results as LDH, like Braakhuis et al., 2023, where it was also found that the WST-1 assay showed lower variability than the LDH assay (7). However, we found a less robust data set seen by the values of CV% (Fig. 9), while in the Braakhuis et al., 2023 study both assays show low interlaboratory variation, indicating a robust and reproducible dataset, this suggested us that the experiment should be repeated with a higher number of samples.

Regarding the over 100% cytotoxicity with the positive control (Triton-x), this could be explained as in the literature, that in the LDH assay, the "100%" positive control assumes maximal lysis, but variations in experimental conditions (e.g., enzyme stability, cell density, and reagent interaction) may lead to higher signal intensities. For example, when the background of the positive control signal is too high/saturated (LDH secretion) could up to the cell concentration is too high or the incubation time is too long (15) and for the future we should consider expand the LPS concentrations or the time of incubation to cause and inflammatory response without membrane damage.

The IL-8 levels indicated that the exposure to clean air did not stimulate IL-8 secretion in Calu-3 cells. The LPS-treated group showed a slight increase in IL-8 levels, though this increase was lower than that observed in the unexposed Calu-3 cells. The decrease in IL-8 levels observed in the LPS-treated group compared to the unexposed cells has not been reported in the literature, particularly in the study by Braakhuis et al. 2023 (7), which involved seven different laboratories. The results from the apical side in that study show that exposure to LPS induces an increase in IL-6 and IL-8 across all laboratories, although the increase was statistically significant in only one laboratory. This discrepancy might indicate that our LPS control needs to be validated adding the cell count to normalize the final data. Additionally, modifications to the incubation time or the concentration of the LPS solution should be considered in future studies. The CV% values showed variability within the treatment groups, particularly in the LPS, corroborating with the inconsistencies or heterogeneity in the inflammatory response.

The cells showed no increase in TNF-α concentration after 24h of XposeALI® exposure or in the LPS control. Therefore, the TNF-α results in our study are aligned with the data produced by 7 laboratories that for the Calu-3 cells, IL-6 and IL-8 could be measured, whereas TNF-α was below the detection limit of 0.01 pg/ml for all

laboratories (7). The next step of this work, it is to develop a co-culture model with CALU-3 and macrophages and the TNF-α will be used as an indicator of the activity of the macrophage cells, knowing the values of this pro-inflammatory cytokine in the monoculture was an important first step.

Despite overall consistency with findings from Braakhuis et al. (2023) (7), significant intra-laboratory variability (e.g., high CV% in LPS and Triton controls) highlights the challenges of standardizing complex *in vitro* systems. The previous study exposed the cells using a different exposure system and some outcomes found here could be attributed to inherent variation of the XposeALI®, therefore better validate those responses, a next step including some experimental refinements (different LPS concentrations, incubation time, higher N, cell counting and anti-inflammatory control) are already being conducted. As an impact of the setting up of the XposeALI® in the Department of Pharmaceutical Technology and Biopharmacy of the UNIVIE, we expect to have a validated method that is compliant with regulatory standards (e.g., OECD, FDA, or ISO), which is crucial for studies with applications in drug development, toxicology, and safety assessments. In summary, validating an XposeALI® method establishes the foundation for scientific integrity, regulatory compliance, and broader application in inhalation toxicology and drug development. It significantly impacts the lab's ability to produce credible, relevant, and high-quality research data.

5. Conclusion

The validated XposeALI® method offers robust alternatives to animal models, aligning with the 3Rs principle in research. As outcomes, the TEER values indicated that the exposure procedure had no significant impact on the barrier integrity of Calu-3 cells compared to unexposed controls. Both LDH and WST-1 assays confirmed that the clean-air exposure procedure was not cytotoxic to Calu-3 cells, as cytotoxicity responses in both testes remained below the threshold of 30%. The pro-inflammatory cytokines IL-6 and IL-8 did not show significant changes following exposure, indicating that the procedure under these conditions did not elicit a pronounced inflammatory response. The high variability in cytokine measurements, particularly in LPS-treated

groups, highlights the need for methodological improvements to ensure reproducibility. Here we demonstrated the utility of the Calu-3 monoculture model for evaluating respiratory toxicology under ALI conditions. The findings confirm that the XposeALI® system can be effectively implemented to study cell barrier integrity and cytotoxicity, with a need for further optimization to improve the controls for the pro-inflammatory after-exposure tests.

6. Perspectives

To advance the current study, the following research perspectives will be prioritized:

I. **Development of a Method for Cell Counting**
   A reliable and standardized method for counting the cells in the inserts after the experiments will be established. This will ensure accuracy in quantifying cell populations, which is critical for assessing the experimental outcomes and comparing results across different conditions.

II. **Expansion of Experimental Sample Size (N)**
   Efforts will be made to increase the experimental sample size to improve the statistical power and reliability of the findings.

III. **Optimization of LPS and Triton Treatments**
   The experimental conditions will be refined by testing various concentrations and incubation times for LPS and Triton. This will help identify optimal parameters for eliciting desired biological responses, ensuring the experimental design is both efficient and effective.

IV. **Diverse Exposure Conditions**
   Beyond exposure to clean air, the study will be expanded to include:

   - An **anti-inflammatory agent**, to explore its potential effects on mitigating inflammation in the experimental model.
   - A **non-toxic nanosized powder (TiO_2)**, to evaluate its impact on cellular responses and assess its safety profile in the context of air-liquid interface exposure.

These directions aim to enhance the study's depth, broaden its scope, and refine the methodologies, paving the way for more comprehensive and impactful research outcomes.

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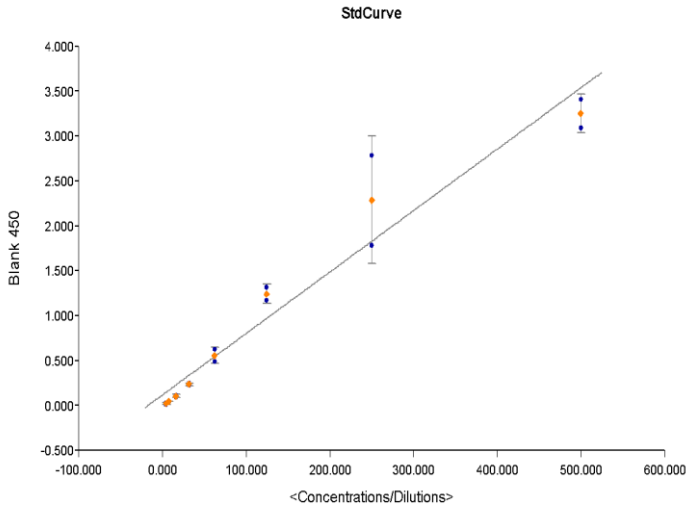
Supplementary material

Calibration curves

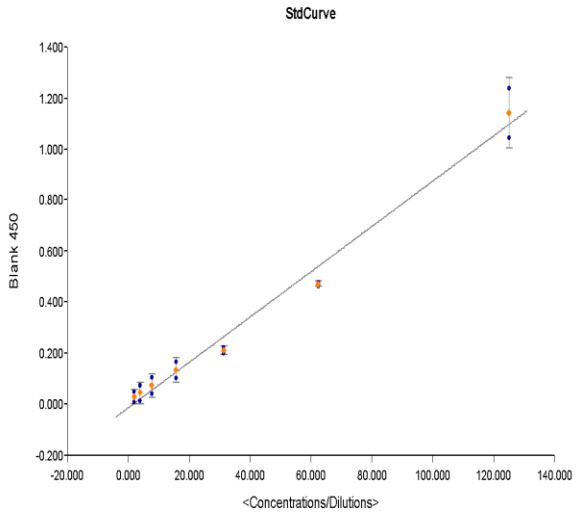
TNF-alfa



	Curve Name	Curve Formula	A	B	R2	Fit F Prob
	StdCurve	Y=A*X+B	0,00684	0,116	0,959	?????

Curve Name	Curve Formula	Parameter	Value	Std. Error	95% CI min	95% CI max
StdCurve	Y=A*X+B	A	0,00684	0,000578	0,00543	0,00825
		B	0,116	0,118	-0,173	0,404

IL-8



	Curve Name	Curve Formula	A	B	R2	Fit F Prob
	StdCurve	Y=A*X+B	0,00887	-0,0135	0,988	?????

Curve Name	Curve Formula	Parameter	Value	Std. Error	95% CI min	95% CI max
StdCurve	Y=A*X+B	A	0,00887	0,000432	0,00776	0,00998
		B	-0,0135	0,0236	-0,0741	0,0471