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Optimization of the CULTEX® radial flow system for *in vitro* investigation of lung damaging agents

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HIGHLIGHTS

- The CULTEX® RFS system is an *in vitro* test system to assess pulmonary toxicity.
- This study was performed to enhance the practicability of the CULTEX® methodology.
- Methodological settings were modified and impact on cell viability was evaluated.
- Open-wall inserts were identified as most suitable for optimal test performance.
- The CULTEX® RFS methodology was successfully optimized.

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ABSTRACT

Exposure of the respiratory tract to airborne particles is gaining more and more importance due to the ubiquitous application of these particles in the field of industry, pharmacy and in daily life. Remarkably, the toxic properties and the underlying pathomechanisms with regard to inhalable substances have been insufficiently investigated so far. Thus, the EU Chemicals Regulation demands toxicological data (including the identification of potential inhalation hazards) for all chemicals placed on the market until 2018 (REACH). This requires extensive, technically complex and expensive inhalation toxicology studies that are usually generated in animal experiments. However, the legislation demands the consideration of the "3Rs" principle. Thus, *in vitro*-based test systems for the assessment of pulmonary toxicity are required. One promising approach to assess acute pulmonary toxicity of airborne particles is the CULTEX® RFS methodology that allows exposure of human lung epithelial cells at the air-liquid interface mimicking the alveolar situation. A prevalidation study showed the general applicability of this method. However, the clean air exposure group, which served as unexposed controls, exhibited some variations with regard to cell viability compared to the incubator control group. The aim of this study was therefore the identification of the possible causes and the improvement of methodological aspects. Several parameters including the general workflow, adjustment of airflow parameters, and cleaning procedures were investigated and adapted. Finally, our results showed the successful optimization of the CULTEX® RFS methodology for clean air exposure of A549 cells. However, although viability data in incubator controls and clean air exposures were equal, a distinct difference in cell morphology was observed that required further optimization. Additional experiments identified that open-wall cell culture inserts with a 2-fold pore density were found to be superior compared to the standard inserts and thus the deciding factor for the improvement of cell morphology. The presented findings are an important step in providing the CULTEX® RFS methodology as a promising alternative method to current *in vivo* testing in inhalation toxicology.

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1. Introduction

The ongoing industrial development and new technical improvements (*e.g.*, nanotechnology) both resulted in rising air pollution that has contributed considerably to the significant increase of pulmonary diseases over the last few decades (BeruBe et al., 2009; Lopez and Murray, 1998). New everyday products (*e.g.*, nanoparticle-containing sprays) have found wide use in industry and electrical, consumer, and medical applications (Yah et al., 2012). Especially products applied in form of sprays or powders must be considered as particularly harmful. Due to the augmented release of harmful particles, *e.g.*, when using nano-sprays for shoe care, cleaning agents, antibacterial sprays, or during technical processing of plastics the risk of pulmonary exposure increased significantly (Singh and Nalwa, 2007). However, the toxic properties and the underlying pathomechanisms of inhalable substances are insufficiently investigated and thus poorly understood (Agrawal and Winder, 1996; Card et al., 2008; Steinritz et al., 2013). Since 2006, the EU chemicals regulation REACH (Registration, Evaluation, Authorization and Restriction of Chemicals, EC No. 1907/2006) demands that existing as well as newly introduced chemicals require a toxicological risk assessment including the identification of potential inhalation hazards (Rovida and Hartung, 2009). Human toxicological *in vivo* data are rare as human intoxication is usually accidental, unpredictable, and uncontrollable. Stratification of a homogeneous cohort is therefore quite challenging. Besides, investigations of lung toxicity in controlled human *in vivo* studies are not feasible due to ethical reasons. Thus, toxicological data are routinely generated in animal experiments. However, animal models may exhibit limitations due to *e.g.*, interspecies differences (Warheit, 1989). Moreover, the legislation demands the consideration of the "3Rs" principle (Replacement, Refinement, and Reduction of animal experiments) and consequently the development, establishment and validation of alternative methods for current animal testing (Combes and Balls, 2014; Costa, 2008; Forbes and Ehrhardt, 2005; Hartung, 2008; Russell and Burch, 1959). Therefore, sophisticated *in vitro* models for the assessment of pulmonary toxicity are required (Worth et al., 2004). A variety of *in vitro* cell culture models have been developed to assess acute pulmonary toxicity of airborne particles. Until now, most of the available *in vitro* cell culture models have been limited to some extent: either exposure is conducted under submerged conditions, leading to potentially changed characteristics of particles, or homogeneous particle distribution is not guaranteed (Aufderheide et al., 2013; Limbach et al., 2005; Raemy et al., 2012). The recently developed CULTEX® Radial Flow System (RFS) is a specially designed *in vitro* exposure system that overcomes these limitations. It enables the homogeneous exposure of cultivated cells at the air-liquid interface closely approximating the inhalation situation *in vivo* and the physiological conditions in the lung (Aufderheide et al., 2013). A prevalidation study has demonstrated the general applicability of the CULTEX® RFS for exposure studies (Steinritz et al., 2013). However, the clean air exposure group, which served as unexposed controls in these experiments, exhibited some variations with regard to cell viability compared to the incubator control group (Steinritz et al., 2013). The reason for that unexpected finding was not solved within the prevalidation study. The aim of this study was therefore the identification of the possible causes and the improvement of the methodological aspects.

2. Materials and methods

2.1. CULTEX® Radial Flow System (CULTEX® RFS)

The CULTEX® RFS methodology was used as described earlier (Aufderheide et al., 2013; Steinritz et al., 2013). In short, the CULTEX® setup consists of three functional units: The CULTEX® Dust Generator (CULTEX® DG), two CULTEX® RFS modules for particle and clean air exposure experiments and the vacuum unit.

Test substances were pressed into powder cakes by a hydraulic press (CULTEX® HyP). Using the CULTEX® DG, the surface of the powder cakes was scraped off by a rotating scraper. The generated particles were transported with a constant air flow to the integrated elutriator. Synthetic air (Linde Gas Therapeutics, Germany) was used as carrier gas and for clean air controls. Particles greater than 8 μm in diameter were trapped within the elutriator whereas particles of lower size were able to reach the CULTEX® RFS exposure module (Aufderheide et al., 2013). In this study, only clean air and no particle exposure experiments were conducted.

2.2. Cell culture

The human lung adenocarcinoma epithelial cell line A549 (CCL-185, ATCC) was used for the experiments. A549 cells were grown in Dulbecco's Modified Eagle's Medium (DMEM) (Biochrom, Germany) supplemented with 10% fetal calf serum (FCS Gold, PAA Laboratories, Germany) and 1% penicillin/streptomycin (Gibco Life Technologies, Germany). This medium is subsequently referred to as "growth medium".

For experiments, A549 cells were seeded in either BD Falcon (PET membrane, 4.2 cm^2, pore size 0.4 μm, pore density $2.0 \pm 0.2 \times 10^6$, BD Falcon, Germany) or Corning Transwell (PET membrane, 4.67 cm^2, pore size 0.4 μm, pore density 4.0×10^6, Corning, Germany) cell culture inserts with a density of 1.2×10^5 cells/cm^2 and cultivated for 24 h under submerged conditions (37 °C and 5% CO_2, further referred to as standard conditions) before exposure at the air-liquid interface.

2.3. Clean air exposure

A549 cells (3 inserts per condition and point in time) were exposed to clean air for 5, 15, or 30 min at the air-liquid interface. Incubator controls (3 inserts) were lifted to the air-liquid interface for 30 min inside the incubator. During clean air exposure or incubation at the air-liquid interface in the incubator, cells were kept in DMEM containing HEPES (25 mM) (Gibco Life Technologies, Germany), supplemented with 1% penicillin/streptomycin. This medium is subsequently referred to as "exposure medium". Afterwards, all cells were again cultivated under submerged conditions in DMEM growth medium until further analysis.

2.4. Cell viability assay

Cell viability after exposure was assessed using the XTT assay. The assay was performed according to the manufacturer's protocol (Roche Diagnostics, Germany). 24 h after clean air exposure the medium in the bottom well was discarded and exchanged with 2.5 mL fresh DMEM growth medium (2.5 mL). 500 μL XTT labeling mixture were added to each cell culture insert and cells were incubated for 2 h under standard conditions. Afterwards, 100 μL of the supernatant were transferred in triplicate from each well to a 96-well plate. Absorbance was measured at 450 nm with a reference wavelength of 630 nm (Tecan Infinite M200 Pro).