Review Article

The Importance of Variations in *In Vitro* Dosimetry to Support Risk Assessment of Inhaled Toxicants

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Abstract

In vitro methods provide a key opportunity to model human-relevant exposure scenarios for hazard identification of inhaled toxicants. Compared to *in vivo* tests, *in vitro* methods have the advantage of assessing effects of inhaled toxicants caused by differences in dosimetry, e.g., variations in concentration (exposure intensity), exposure duration, and exposure frequency, in an easier way. Variations in dosimetry can be used to obtain information on adverse effects in human-relevant exposure scenarios that can be used for risk assessment. Based on the published literature of exposure approaches using air-liquid interface models of the respiratory tract, supplemented with additional experimental data from the EU H2020 project "PATROLS" and research funded by the Dutch Ministry of Agriculture, Nature and Food Quality, the advantages and disadvantages of different exposure methods and considerations to design an experimental setup are summarized and discussed. As the cell models used are models for the respiratory epithelium, our focus is on the local effects in the airways. In conclusion, in order to generate data from *in vitro* methods for risk assessment of inhaled toxicants it is recommended that (1) it is considered what information really is needed for hazard or risk assessment; (2) the exposure system that is most suitable for the chemical to be assessed is chosen; (3) a deposited dose that mimics deposition in the human respiratory tract is used, and (4) the post-exposure sampling methodology should be carefully considered and relevant to the testing strategy used.

Plain language summary

The impact of airborne pollutants on human health is determined by what pollutant it is, how much we breathe in, for how long and how often. Testing in animals is cumbersome and results may not reflect human health impacts. Advanced cell models of the human lung allow prediction of the health impact of many different exposure scenarios. Here, we compare different models and exposure methods and provide criteria that may assist in designing experiments, interpreting the results, and thus assessing the risks posed by airborne pollutants. We recommend (1) determining what information is needed to plan the experiment, (2) choosing an exposure method that is suitable for the pollutant of interest, (3) determining the amount of pollutant that interacts with the human lung, to relate this to realistic deposition in the lung, and (4) considering the time between the exposure and measurement of the effect.

1 Introduction

Real-life inhalation exposures are dynamic and may vary from a brief peak exposure of a few minutes to a continuous 24/7 exposure from environmental air. Exposures may be daily, once a week or less frequent. Exposures include single daily peak exposures (e.g., during use of a spray such as a deodorant) or multiple daily peak exposures such as vaping or smoking tobacco cigarettes. In addition, sources of exposure to a chemical may vary, leading to several possible exposure scenarios for one chemical or particle. For example, exposure to formaldehyde may be occupational (production and occupational use as, e.g., an industrial disinfectant or preservative in funeral homes and medical labs), or via indoor air (e.g., resulting from combustion processes such as heating, cooking, or tobacco smoke, or emanating from building materials).

The assessment of human health risks from the possible variations in inhalation exposures can be challenging when based on *in vivo* toxicity data. Such data are often limited to information from an animal experiment with a standard exposure regimen such as 6 h/day for 5 days per week over 28 days or 90 days as prescribed in OECD Test Guidelines 412 and 413, respectively. If epidemiological data are available, the exposure generally is expressed as a rough estimate, i.e., as ppm-years, or as an inhaled average daily

dose. Based on such data, temporal characteristics within a day and among days cannot be accounted for, and health risks for inhalation exposures of varying durations are assessed by linear extrapolation to the exposure conditions of interest, i.e., application of Haber's Law. This refers to the assumption that with inhalation exposures health effects are related to the product of exposure concentration and duration (i.e., C×t), irrespective of the dosing rate. However, this assumption has been challenged (Bos et al., 2021; Atherley, 1985; Belkebir et al., 2011; Kuempel et al., 2015). For instance, within emergency response planning, acute exposure guideline levels (AEGLs) are derived for different levels of health effects ranging from discomfort to death. AEGLs are derived for single exposures lasting from 10 min to 8 h. For many substances, it appears that Haber's Law does not apply by default, but that extrapolation over time is best described by $C^n \times t$, in which the exponent n depends on the acute health effect and generally varies between 1 and 3 (ten Berge et al., 1986). In addition, for local effects on the respiratory tract, it is considered that the environmental concentration is the driving force, and exposure duration does not play a major role (ECHA, 2012). However, as reviewed by Bos et al. (2021), extrapolation over time for local respiratory effects also appears to be more complex.

Currently, many efforts are being invested towards the transition to animal-free innovations in chemical risk assessment, i.e., the development and adoption of new approach methodologies (NAMs) (Escher et al., 2022; ECHA, 2016). Also, for human health risk assessment of inhalation exposures, NAMs offer promising possibilities from a scientific, economic, and ethical perspective. Scientifically, the added value of NAMs in studying inhalation exposure may be to better address the temporal characteristics in inhalation exposures than can be done *in vivo*. For example, the duration of cellular or tissue contact with an inhaled substance can be better controlled and determined *in vitro* than *in vivo*. This helps to better address multiple exposure scenarios with varying exposure durations and allows identification of the appropriate dosimetry relevant for the human situation. Where animal experiments are time- and resource-consuming, multiple combinations of exposure duration, frequency, and concentration can be tested with *in vitro* methods in a timely and more economic manner. In this way, it is possible to move away from animal studies and at the same time provide better insight into the relationship of adverse effects as a function of exposure conditions, i.e., concentration, duration, and frequency, which will help to address potential health risks from a broad variety of human-relevant exposure scenarios.

It is noted that temporal issues in testing designs may apply to *in vitro* methods in general. The present paper focuses on *in vitro* methods that address local effects on the respiratory tract and their relevance to support the assessment of human health risks from inhalation exposures. It aims to describe the impact of varying exposure conditions – not only regarding concentration but also duration and frequency – on the toxicity outcome. It discusses the efforts that have been made to address these exposure characteristics using *in vitro* methods with primary cells or cell lines of human origin representing the different compartments of the respiratory tract. The exposure characteristics and the design of *in vitro* methods differ significantly from those of respective *in vivo* methods. This includes both the exposure and the post-exposure period until toxicity measurements are performed. These differences are discussed in light of their relevance and applicability for human risk assessment. Where possible, guidance is provided for the design and performance of *in vitro* experiments to generate the most optimal data suitable to assess human health risks of respiratory tract effects relevant to real-life inhalation exposures.

2 Methods and data

Literature search

In order to obtain examples that illustrate the impact of the varying exposure conditions (i.e., concentration, duration, and frequency) on the *in vitro* toxicity outcome and its relevance for safety assessment of inhalation exposures, a literature search was performed. The present paper is not a comprehensive, systematic overview of the available data, but is narrative in nature.

A literature search for publications on *in vitro* methods with exposure of respiratory epithelial cells to chemicals (i.e., gases, vapors, or aerosols[1]) via the air liquid interface (ALI) was performed using the database embase.com starting from 2015 up to and including 2022. The search strategy initially included search terms for air exposure systems and cell models representing the respiratory system. The strategy focused on exposure (to chemicals) and was further refined with search terms for parameters of cell viability, barrier formation, cytokines/chemokines, oxidative stress, and some specific parameters such as morphology, cilia, or mitochondrial function. The detailed search strategy is presented in Table S1[2].

The obtained records were first evaluated based on the abstract, and original research papers describing exposure (to chemicals) of respiratory epithelial cells at the ALI were selected for full text review. Records were considered relevant if the study design included quasi-ALI, aerosol exposure, closed chamber exposure or continuous exposure; if different exposure concentrations, durations or frequencies were applied; and if effects in relation to these variations were observed. Studies that included read-outs of at least one toxicity endpoint at different timepoints after exposure in the study design were considered relevant as well for the purpose of the review. Table S2[2] presents an overview of the relevant *in vitro* methods obtained.

It was outside the scope of this review to specify the currently available respiratory tissue models that mimic the different compartments such as the human airway and lung parenchyma. This has been extensively described elsewhere (Nichols et al., 2014; Ahookhosh et al., 2020; Miller and Spence, 2017; Hiemstra et al., 2018; Rothen-Rutishauser et al., 2023). However, as some of the endpoints discussed below are cell- and compartment-specific, a brief overview is provided.

[1] A heterogeneous mixture of particles together with the gas or gas mixture surrounding them.

[2] doi:10.14573/altex.2305311s1

Tab. 1: Definitions of exposure concentration, exposure duration, exposure frequency and post-exposure period for the different exposure systems

	In vitro				*In vivo*
	Quasi-ALI (non-volatile chemicals[a])	**Aerosol exposure (aerosols only[a])**	**Closed chamber (gases and vapors)**	**Continuous exposure (gases/ vapors or aerosols[a])**	
Exposure intensity	Quantity of test compound applied in relation to surface area	Quantity of test compound deposited in relation to surface area	Concentration of test compound in the air	Concentration of test compound in the air and for aerosols also the deposited dose	Concentration of test compound in the air (and, if available, also retained dose)
Exposure duration	Time it takes to apply dose	Duration of aerosolization	Duration in the closed chamber	Duration of exposure to a test atmosphere flow	Duration of exposure to a test atmosphere flow
Exposure frequency (within 24 h)	Number of applications within 24 h	Number of aerosolizations within 24 h	Number of exposures within 24 h	Number of exposures within 24 h	Number of exposures within 24 h
Post-exposure period	Time between applying the last dose and measurements	Time between end of aerosolization and measurements	Time between removal from chamber and measurements	Time between end of exposure and measurements	Time between end of exposure and measurements

[a] This method can be used for exposures to mixtures, however, processes like aerosol aging and evaporation of volatiles should be considered.

Human primary cultures of nasal, tracheal, and bronchial epithelial cells, which can be derived by nasal brushings or biopsies, can re-differentiate under ALI conditions into pseudostratified epithelium. In this way, they retain important properties of differentiated airway epithelial cells such as growth in polarized monolayers with extensive tight junction belts and cilia (Stokes et al., 2014; Forbes and Ehrhardt, 2005; Steimer et al., 2005). The cultures can be used for long-term experiments (chronic/repeated aerosol exposures) over several weeks to months and allow the study of mucus production and mucociliary beating frequency. Immortalized cells, i.e., cell lines, can be cultured at ALI for exposure to airborne compounds. Although they have their advantages, especially in terms of ease of handling and homogeneity, they resemble the human airway epithelium less well than differentiated primary human epithelial cells. Recently, progress has also been made in mimicking the alveolar region, and primary cells from lung biopsies or immortalized cell lines have been used to study the effects of aerosols. Some of the models can also be kept stable at the ALI to study repeated exposure to aerosols (Silva et al., 2023; Barosova et al., 2020b; Blank et al., 2006).

Original data

Table S3[2] describes an exposure scheme for volatile chemicals, which we applied for dichlorvos exposure of ALI-cultured Calu-3 bronchial cells. As no effects were found, the data are not described further. Supplementary files 2[3] and 3[4] contain original data on continuous exposure to crystalline silica (also referred to as quartz), e.g., DQ_{12}, at the same total dose but for different exposure periods and quasi-ALI exposures to benzo[a]pyrene, chlorpyrifos, and nicotine using the same dose rate (concentration x duration) in different scenarios. The quasi-ALI exposure method is described below and in supplementary file 2[3]. Detailed information on the experiments can be found in the supplementary files[3,4].

3 Exposure-related effects

Terminology describing *in vitro* exposures varies and differs from that used for *in vivo* exposure. To compare results of different *in vitro* methods with each other and to verify their relevance for human health risk assessment of *in vivo* exposures, a clear and consistent terminology is needed.

In vivo exposures generally are defined by the airborne concentration of the chemical and the exposure duration. Exposure duration then refers to the time period during which a chemical is inhaled. Dissolution in the epithelial lining fluid or deposition of a chemical in the respiratory tract will continue during exposure until the exposure has ceased. Following exposure, the chemical will still be present until it has been cleared. The retained or deposited dose for *in vivo* exposures is often unknown, and health effects are related to the external exposure characteristics.

For *in vitro* exposures, the exposure may be described differently. Concentration may refer to the concentration in air (in case of gases and vapors), to the concentration in the aerosols that are deposited onto the cells, or to the concentration in aerosols that are directly applied to the cells. The exposure of cells is expressed as applied dose or, preferably (Schmid and Cassee, 2017), deposited dose, e.g., mass per surface area. For *in vitro* methods, the depos-

[3] doi:10.14573/altex.2305311s2

[4] doi:10.14573/altex.2305311s3

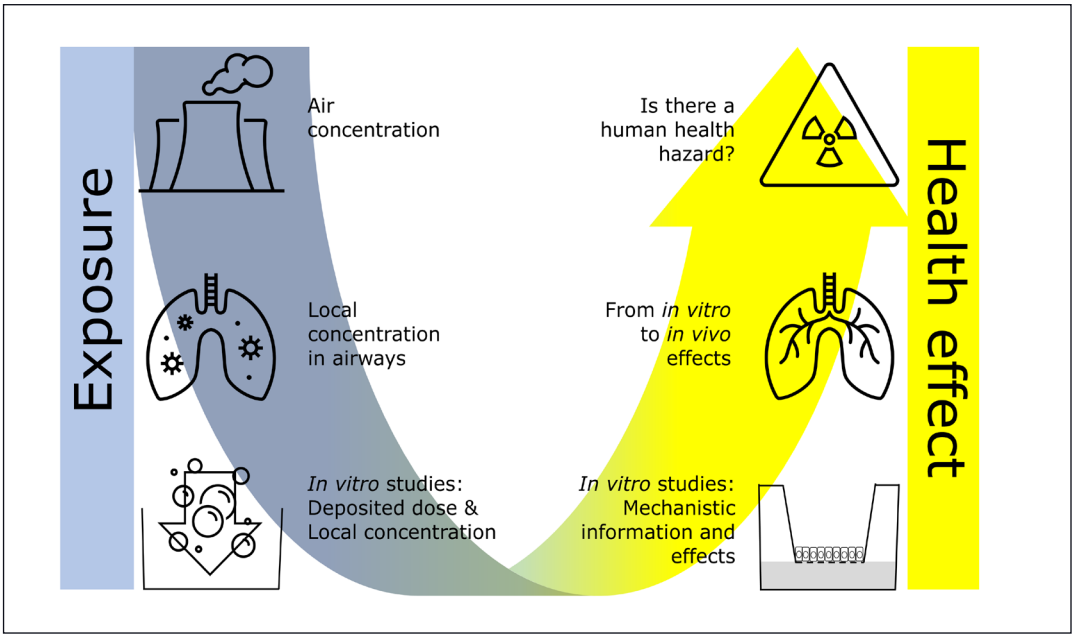

Fig. 1: Overview of the relation between exposure and health effect, going from air concentration to local concentration in the airways to the *in vitro* deposited dose or local concentration
This deposited dose can be used to study *in vitro* biological effects and relate this to effects that would be expected *in vivo* to identify a human health hazard.

ited dose is usually applied over a relatively short period of time followed by a post-exposure period. The term "exposure duration" is used interchangeably and subjectively, but often refers to the period during which the cells are in contact with the test material, in contrast to its use in *in vivo* exposures. In the present paper, this timeframe is referred to as the "post-exposure time", while exposure duration is explicitly used for the time period of application, similar to its use for *in vivo* exposures. Further, a chemical may be applied more frequently, i.e., more than once, within an *in vitro* experiment, for which we distinguish two situations. In case multiple depositions take place within 24 h, the number of depositions is referred to as frequency of exposure within 24 h. The amount of chemical deposited within 24 h then is the total deposited dose. If multiple depositions are divided over two or more periods of 24 h, this is referred to as frequency of exposure beyond 24 h or repeated exposure, where units of time are 24-h time periods (Meldrum et al., 2022). This is further described in Table 1.

Figure 1 gives a schematic overview of *in vivo* and *in vitro* exposure and health effects. We use "exposure intensity" to describe the quantity of chemical as "exposure concentration" can be confusing for quasi-ALI and cloud exposures. These definitions are inspired by human exposure, where the exposure duration is defined as the time the test compound is inhaled. Once inhaled, the compound comes into contact with the cells of the respiratory tract, where the local exposure is expressed as mass per surface area. For all exposure methods, the deposition is the quantity of test compound on the apical side of the cell culture, and repeated exposure is the number of days (i.e., 24-h periods) on which the exposure is repeated.

3.1 Exposure systems

Since the terminology used may differ among exposure systems, we first introduce the exposure systems that are most commonly used in the *in vitro* method studies discussed in this section. Table 2 presents an overview of the advantages and disadvantages of these exposure systems for risk assessment.

3.1.1 Quasi-ALI

Exposure of cells from the respiratory tract cultured at the ALI can be done relatively easily by applying a small volume of test solution to the apical side of the culture; this is called "quasi-ALI exposure" or "droplet exposure" or "pseudo-ALI exposure" (Endes et al., 2014). This method allows testing of a larger range of chemicals and concentrations compared to air exposure and can be done in laboratories that do not have an exposure system for airborne chemicals at hand. However, quasi-ALI is not suitable for all test substances and is most often used for soluble and insoluble particles and for mixtures, i.e., environmentally collected samples. Jeong and colleagues showed that quasi-ALI exposure allows testing of many compounds in different quantities in a standardized approach (Jeong et al., 2019). To achieve different doses, the chemical is applied in varying quantities to the apical side. As the chemical is applied by pipetting, the duration of application (the exposure duration, as defined in Tab. 1) is very short. However, application of multiple exposures is easily possible with this method, although dilution issues of both the exposed agent as well as the biological factor measured (e.g., an inflammatory mediator released by the cells) may have to be considered. After a post-exposure period, the cells are harvested, and parameters of interest are measured. To use these data for risk assessment, the quantity of test material in the droplet relative to the cell surface area should be related to inhaled doses.

3.1.2 Aerosol exposure and closed chamber exposure

A simple way to expose ALI-cultured cells to airborne compounds is to nebulize the test compound and let it settle on the cells. Many research groups use the commercial VITROCELL® cloud system for this purpose (Bannuscher et al., 2022; Nair et al., 2020; Ba-

Tab. 2: Advantages and disadvantages of the different *in vitro* exposure systems for risk assessment (including derivation of health-based guidance values)

	Quasi-ALI	Aerosol exposure (aerosols only)	Closed chamber (gases and vapors)	Continuous exposure (gases/vapors or aerosols)
Advantage	– Allows testing of a wider range of doses without the technical need for an air exposure system. – Allows exposure of multiple cell cultures simultaneously (high n) – Allows application of relatively high concentrations – Limited quantity of chemical needed for exposures	– Relatively easy way of air exposure – Even distribution of aerosol on cells – Limited quantity of chemical needed for exposures	– Relatively easy way of air exposure – Allows exposure of multiple cell cultures simultaneously (high n)	– Mimics *in vivo* exposure most closely – Suitable for all type of chemicals (gases, vapors, aerosols) – Allows variation of concentration, duration, and frequency
Disadvantage	– Exposure duration fixed – Not suitable for gases and vapors – Comparable to *in vivo* intratracheal instillation and therefore possibly less relevant for human situation	– Exposure duration fixed (possibility for repeated exposures) – Not suitable for gases and vapors – Low throughput	– Technical expertise required – Not suitable for aerosols – Less suited to apply different exposure concentrations – Low throughput when testing multiple concentrations	– Complex exposure method; technical expertise required – Low throughput – Deposited dose is relatively low – Relatively large quantity of chemical needed for exposures

rosova et al., 2020a), in which a single aerosolization takes about 15 min. A closed chamber can be used for exposure to vapors and gases (Thimraj et al., 2019). Here, the cells are placed in a closed chamber with a fixed concentration of vapor or gas, without airflow and without suppletion of losses in test compound. The choice of the method depends on the test compound. Cloud exposure works quite well for particle or stable chemical suspensions, but compounds that are instable in aqueous solutions will be degraded before they reach the cells.

3.1.3 Continuous exposure

A further exposure method is continuous (or dynamic) exposure, in which the cells are exposed to a continuous flow of test atmosphere (Polk et al., 2016). Continuous exposure allows variation of concentration, duration, and frequency. It is more difficult to determine deposition for continuous exposure, but information on deposition should be collected or measured for continuous flow systems. As mentioned in Table 2, this method is suitable for all kinds of substances, in contrast to the other exposure methods described. Complex aerosols, like combustion engine emissions or tobacco smoke, and other mixtures of compounds may change in the process due to aerosol aging or temperature changes. Continuous exposure of the cells most closely resembles human exposure to complex aerosols concerning their composition and particle size distribution. Still, one should be aware of changes in aerosol dynamics during exposures to such complex mixtures.

3.2 Deposited dose

A direct comparison between human exposure and *in vitro* concentration based on air concentrations may not always be representative for exposure of human respiratory epithelial cells. Instead, the deposited dose is used for this purpose. For vapors and gases, *in vitro* deposition can often be determined from the concentration in the air at the site of the cells, e.g., using chemical analysis. For aerosols, the *in vitro* deposited dose can be determined by chemical analysis. For aerosol or continuous exposures it is also possible to determine the *in vitro* deposited dose by weighing, with a Quartz microbalance, or to model the deposited dose using *in silico* models (e.g. the Multiple-Path Particle Dosimetry (MPPD) model from Applied Research Associates, Inc.). However, for complex mixtures measuring the deposited dose is challenging and may not be feasible, although chemical analysis and deposited mass can give insight into the actual exposure. For cloud exposures, the *in vitro* deposited dose can be quite accurately calculated from the quantity aerosolized and the total surface area of the system (including the spaces between the inserts).

4 Exposure intensities

A quasi-ALI setup has been used to study exposure intensity-related effects of chemicals or particulate matter by measuring endpoints such as cell viability, reactive oxygen species (ROS)

production, transepithelial electrical resistance (TEER), FITC-dextran paracellular flux, inflammatory markers, intracellular glutathione, and gene expression (Zhang et al., 2020; Ma et al., 2021; Jang et al., 2021; Kim et al., 2016; Willoughby, 2015; Meldrum et al., 2022; McGee Hargrove et al., 2021; Baiocco et al., 2021; Welch et al., 2021). This shows that quasi-ALI exposure is a suitable method to find exposure intensity-related effects of exposure.

To obtain different deposited doses (or varying exposure intensity) with a cloud system, the concentration of the solution that is aerosolized can be varied (Wang et al., 2021; Stoehr et al., 2015; Nair et al., 2020; Hu et al., 2020; Fizeşan et al., 2019; Chary et al., 2019; Binder et al., 2021; Bessa et al., 2021), the volume of the solution can be varied (Schmid et al., 2017; He et al., 2020; Di Ianni et al., 2021), or the exposure can be repeated sequentially to increase the deposited dose (Barosova et al., 2020a). The approach chosen depends on the test compound. For example, particles in suspension will more easily aggregate at a higher concentration compared to a lower concentration, implying that dilution of a suspension may result in a different exposure, for example in terms of particle number and size distribution. Most researchers describe their approach to obtain different exposure intensities, although it is not always clearly stated whether different exposure intensities were achieved by aerosolizing solutions of different concentrations or by repeated aerosolization (Friesen et al., 2022a,b). For testing the effects of (nano)particles, usually a single concentration is chosen to prepare the dispersion, and the number of nebulizations is adapted to achieve different deposited doses. Irrespective of the method used to achieve different exposure intensities, concentration-related effects have been described for parameters such as cell viability, inflammatory response (mostly interleukin (IL)-8 and IL-6), or gene expression parameters. Exposure via nebulization has therefore been shown to be a good method to obtain concentration-related responses. None of the reports describe the variation of exposure duration or exposure frequency (within 24 h) in addition to the exposure intensity.

Exposure concentration or exposure duration can be varied to obtain different doses for continuous exposure. Varying exposure concentrations is technically more complicated as it requires dilution of test atmospheres. This exposure method can be applied for aerosols (Wang et al., 2019; Svensson et al., 2016; Sayes and Singal, 2021; Kooter et al., 2017; Jonsdottir et al., 2019; Ishikawa et al., 2018; He et al., 2021) and for vapors or gases (Verstraelen et al., 2021; Sayes and Singal, 2021; Moreau et al., 2022; Méausoone et al., 2019, 2021; Dwivedi et al., 2018).

However, most studies do not vary the exposure duration or frequency within 24 h in addition to the concentration. The study by Ishikawa et al. (2018) is an exception, as they not only varied the number of cigarettes smoked (1 or 2 cigarettes simultaneously), but also the dilution of the smoke (no dilution, 1 or 3 L/min dilution) and the duration of smoking (once or twice 2 cigarettes), thereby creating a variation in both concentration as well as duration. They described concentration-related effects on cell viability, cytokines (IL-8, IL-6, monocyte chemoattractant protein (MCP)-1), gene expression, including heme oxygenase (HMOX)-1, and protein expression. However, it would have been interesting if they had included a scenario that would have resulted in the same dose over 24 h to assess the effects of concentration in relation to duration.

5 Exposure durations

In the following examples, different deposited doses were achieved by varying the exposure duration in a continuous exposure system while the aerosol concentration (and flow) was kept constant. More detailed information on the experiments can be found in Table S2[2].

Different exposure durations were applied by Jing et al. (2015) (2 or 4 h), Medina-Rey et al. (2020) (1 or 4 h) and Tilly et al. (2020) (15, 30, 45 or 60 min) when investigating nanoparticles and -fibers. Duration-related effects were noted for viability, ROS production, inflammatory markers, and DNA strand breaks.

Similarly, Ji et al. (2019) and Klein et al. (2017) varied the exposure duration to deposit different doses at constant aerosol flow rate and (nano)particle aerosol concentration. Duration-related effects on inflammatory markers and cellular (oxidative) stress were noted (Klein et al., 2017; Ji et al., 2019). However, the exposure durations of these two studies were quite short, i.e., 1, 2 or 3 min with 6- and 24-h post-exposure read-out timepoints for Ji et al. (2019), and 1 min 8 s, 2 min 17 s or 6 min 52 s with 6-, 24-, or 48-h post-exposure read-out timepoints for Klein et al. (2017).

Jeannet et al. (2016) applied a similar approach by varying the exposure duration from < 1 min up to 1 h. Deposited particles not attached or taken up by cells were removed after 4 h by washing the apical cell surface. Exposure duration-related effects were noted for various inflammatory markers.

For testing of cigarette smoke and/or e-cigarette aerosol, Neilson et al. (2015) and Bishop et al. (2019) varied the exposure duration at fixed aerosol concentration. Neilson et al. (2015) used exposure durations of 1 to 6 h, whereas Bishop et al. (2019) used various exposure durations up to 1 h. Exposure duration-related effects were noted for viability, TEER, and cilia function (Neilson et al., 2015; Bishop et al., 2019). In the example of Ishikawa et al. (2018), as presented in Section 4, also the exposure duration was varied. They found a difference between a single exposure (5 min) to 2 cigarettes and a double exposure (10 min), indicating that exposure duration does contribute to the observed effects.

Variation of the exposure duration is easily applied under continuous exposure conditions, as presented in the examples above. Here, the exposure duration is defined as the period during which the cells are exposed to a test atmosphere flow. For both quasi-ALI as well as aerosol exposure the exposure duration (i.e., the time it takes to apply the dose and the duration of aerosolization, respectively) is fixed. Varying the exposure duration is feasible for static exposure of vapors and gases in a closed chamber. However, such examples were not found in the literature search. Examples as presented above were restricted to the continuous exposure method and, regarding the type of test item, limited to aerosols.

These examples indicate duration-related effects for multiple parameters upon exposure to many different test items. Such ef-

fects are in most cases described by the authors as effects in relation to the deposited dose (mass per cm^2) rather than in relation to the exposure duration. Importantly, in some cases the terms dose and concentration are used interchangeably when describing the mass per surface area, with such effects being presented as a concentration-related effect. This is considered incorrect.

As in the above examples variation in exposure duration was applied to obtain different deposited doses, for non-volatile substances the exposure duration and deposited dose are dependent variables and linearly related to each other. This makes it difficult to clearly distinguish between these variables in relation to the outcome of the *in vitro* method. In case of very short exposure durations relative to the post-exposure time-period, the difference in exposure duration may be negligible. It is also noted that the observed effects can be the result of a combination of exposure duration and the selected post-exposure read-out timepoint.

Variation in exposure duration under continuous exposure conditions is applied within the hour-range but also in the minute-range. For short exposure durations, the precision of the dosing time is then an important aspect. Nevertheless, the examples show that continuous exposure is a suitable method to explore exposure duration-related effects.

In the above-mentioned studies, exposure duration was the only variable used in the study designs to obtain a different dose. An exception to this is Anthérieu et al. (2017), who applied two exposure durations within one day as well as two frequencies. This example is presented in more detail in Section 6.

Supplementary file 2[3] describes an experiment in which the same total dose of DQ12 particles (about 1.5 µg/cm^2) was applied for 1 day, 3 days or 3 weeks (5 days per week, 4 h/day). According to our definitions in Table 1, this is a repeated exposure over multiple days and not a variation of exposure frequency or duration. Nevertheless, this is the only experiment we know of that used the same total dose for each of the exposure periods. Results show that exposure to a high exposure intensity for a short time period results in more pronounced effects than exposure to a low exposure intensity for a longer period. In addition, responses were different, with an increased IL-6 and IL-8 response on day 1 and a tumor necrosis factor α (TNF-α) response on day 3. As the effects are dependent on the exposure duration, these results indicate that extrapolation of concentrations to human-relevant exposure scenarios, as needed for risk assessment, cannot be achieved by simple extrapolation of the total dose.

6 Exposure frequencies

Variation of exposure frequency was applied in the following examples. More detailed information on the experiments can be found in Table S2[2].

In addition to varying the exposure duration within one day, Anthérieu et al. (2017) also varied the frequency by applying single continuous exposures of 8 or 48 min and 6 separate 8-min exposures within one day (with 1 exposure/h). Their study design allows for comparison of single versus multiple exposures at different frequency and different total dose when considering a single 8-min versus 6 separate 8-min exposures. Furthermore, by varying both the exposure duration as well as the exposure frequency, they designed scenarios that resulted in the same total dose (i.e., repeated 6×8 min exposure versus single 48-min exposure). They found that, in general, changes in gene expression and cell viability effects were greater after a single 48-min exposure than after multiple exposures (6×8 min). The authors suggested that an adaptive (recovery) response might explain the differences, as for the multiple exposure (within 24 h) scenario cells were only exposed 6 times for 8 min/h and a single 8-min exposure was shown to have minor effects (Anthérieu et al., 2017).

A special case is presented by Chortarea et al. (2015), who compared single exposure to two different scenarios for repeated exposure to multi-walled carbon nanotubes (MWCNT) (via the VITROCELL® cloud system). Cells were exposed three times over one day or three times in three days, applying different frequencies. Although for the single exposures three different concentrations (low, mid and high) were aerosolized, only the high aerosol concentration was selected for the repeated exposures. This allows for a comparison of single versus repeated exposure and thus varying the exposure frequency (at similar exposure concentration and duration, and thus different total dose). Interestingly, this study design also allows for a comparison of different repeated exposure scenarios with different exposure frequencies at similar total dose. Regarding the latter, it was shown that at similar total dose, higher MWCNT uptake was observed in the 3-d repeated exposure compared to the 1-d repeated scenario. The study authors suggested that the cells need more time to internalize MWCNTs at the selected doses, thus indicating the internalization to be more efficient at three days of exposure. However, no significant effects on cytotoxicity, cell morphology, pro-inflammatory markers or glutathione levels were observed (Chortarea et al., 2015).

Different exposure frequencies refer in this case to variations within 24 h. Applying different exposure frequencies to ALI-cultured cells is practicable for all four different exposure methods presented in Table 1. This can be achieved by varying the number of times a dose is applied for quasi-ALI or varying the number of exposures for continuous exposure. Regarding static exposure, this can be achieved by varying the number of aerosolizations for aerosols or varying the number of exposures in a closed chamber for gases and vapors.

Though varying the exposure concentration and exposure duration is in most cases aimed at obtaining different doses, this does not always apply to variation in exposure frequencies. Especially regarding continuous exposure, different exposure frequencies are usually applied to create specific exposure scenarios. The exception is aerosol exposure and quasi-ALI exposure, as here varying the exposure frequency can be used to obtain different doses in case varying the exposure intensity is limited by physical-chemical properties for a specific test item.

The example of Anthérieu et al. (2017) nicely shows that by varying the exposure frequency and duration at similar doses, intervals without exposure are introduced, which might affect the cell responses.

7 Endpoint measurements

Many studies measure biological parameters over time, starting just after exposure and at different time points after exposure. For many *in vitro* methods this is an advantage over *in vivo* models, where it is more complicated to add sample times. This section aims to highlight the importance of selecting the post-exposure period for different measurements, i.e., *in vitro* bioassays.

The aim to predict adverse health effects in a whole organism, based on observed *in vitro* responses, has resulted in a program on the development of adverse outcome pathways (AOPs) launched by the OECD (2017). This framework takes human epidemiology, *in vitro*, and *in vivo* data into consideration to describe causally connected key events (KEs) starting from a molecular initiating event (MIE) and resulting in a specific adverse outcome (AO) (Halappanavar et al., 2020, 2021). In the field of inhalation toxicology, relevant KEs leading to the AOs of inflammation-induced fibrosis are mainly used for hazard characterization[5] (Halappanavar et al., 2020, 2021). Some of the key events such as cell viability, inflammatory markers, and barrier integrity are described below as they have been investigated in the papers found in the literature research. This selection is far from complete, and other endpoints can be included in a study depending on the expected outcome in animals or humans. The focus is on the differences in response between different post-exposure periods; more detailed information on the experiments can be found in Table S2[2].

7.1 Cell viability assessment

The results of Fizeşan et al. (2019) indicate that high concentrations of insoluble particles may induce cell damage after shorter post-exposure periods than lower concentrations. Therefore, the peak in response is dependent on the concentration tested. Medina-Reyes et al. (2020) also tested insoluble particles and fibers and found an increase in cytotoxicity during the post-exposure period. These insoluble particles are likely to stay on the apical side of the cell culture or are taken up by the cells, which means that the cells stay in contact with the particles leading to cytotoxicity at later time points. Ren et al. (2022) and Wang et al. (2021) both found a difference in LDH response on the apical and basolateral side. This shows the importance of selecting both the relevant post-exposure period and sample location.

7.2 Epithelial barrier integrity

TEER is often used to monitor epithelial cell growth and differentiation. Exposure to chemicals can damage the cell layer, resulting in a lower TEER. Welch et al. (2021) and Ren et al. (2022) found exposure-related effects on TEER. However, epithelial TEER may also change over time, as shown by Ma et al. (2021). This illustrates the importance of time-matched controls.

7.3 Cytokine/chemokine production

Cellular cytokine production and release into the cell culture medium reflects the accumulated cytokine production over time if medium is not changed and cytokines are not degraded. Stoehr et al. (2015) and Dwivedi et al. (2018) found increased cytokine (IL-8 or matrix metallopeptidase (MMP)-9) secretion after longer post-exposure periods. After longer post-exposure periods, cytokine production can return to baseline levels, as found by Welch et al. (2021). Some exposures can, however, still lead to increased cytokine production after a longer period (Baiocco et al., 2021; Meldrum et al., 2022).

Supplementary file 3[4] describes a quasi-ALI exposure with the same dose rate in different scenarios. The results show that some effects may only occur above a specific dose, or they may only occur at or after a specific time after exposure. Although extrapolation of a concentration to another exposure duration is common in risk assessment, these data show that a different exposure scenario (at the same total dose) can lead to very different effects.

Finally, in addition to the timing of sampling, the location of sampling is also important for ALI-cultured cells. Cytokine responses on the apical side can be different from those on the basolateral side, as shown by several researchers (Wang et al., 2021; Fizeşan et al., 2019; Dwivedi et al., 2018) and in supplementary file 3[4]. It is important to consider on which side of the cell culture the samples are taken, as this may affect the readout. For lung co-culture models, one should consider whether cytokines released from macrophages – or other cells that are added to the apical side of the epithelial cells – can reach the basolateral sampling side (or *vice versa*). Especially in case of a tight barrier and high TEER, cytokines may be retained on a specific side of the epithelial cells.

7.4 Gene expression changes

Gene expression changes reflect a momentary effect and can be used to differentiate between early and late responses to the exposure for a deeper mechanistic insight (Thimraj et al., 2019; Klein et al., 2017; Fizeşan et al., 2019). Friesen et al. (2022b) found a time-dependent response of inflammatory markers (IL-1α, IL-1β, TNF-α, IL-6 and IL-8) after exposure to crystalline quartz (Min-U-Sil5). Kim et al. (2016) found most response in the expression of MMP-2, MMP-9, TMP-1 and TMP-9 at the longest post-exposure period (24 h). The measured genes are involved in the development of fibrosis, which may explain the later response after exposure. Mathis et al. (2015) followed gene expression responses from 2 to 48 h post exposure and found an oxidative stress response shortly after exposure and an inflammatory response at later time points. Supplementary file 2[3] also shows the differences in gene expression for different post-exposure time points (and concentrations). These data highlight the importance of mechanistic information in selecting the post-exposure time point.

7.5 Genotoxicity

Binder et al. (2021) and Medina-Reyes et al. (2020) found no dose-related effects on DNA strand breaks upon different post-exposure periods. The absence of a dose-related response may be due to the different processes that are ongoing, like induction of DNA damage and DNA repair, and in some cases also continued

[5] https://aopwiki.org/aops/173

exposure with less possibility for recovery. This shows the importance of insight into mechanisms of adverse effects to select the post-exposure time point.

7.6 General considerations to select a post-exposure period

The paragraphs above reflect on selecting post-exposure periods for specific endpoints. Specifically for air exposures, it should be considered when the response begins. Some effects may start at the initiation of exposure. When the exposure duration is relatively long, in case of continuous exposure or static exposures to gases and vapors, the peak of the response may occur during the exposure period. This should be considered when designing an experiment. For quasi-ALI, cloud exposures, and continuous exposures it should be considered whether and when the cells are washed on the apical side. Insoluble compounds will stay on the apical side, resulting in ongoing contact between the chemical and the cells. In human lungs, such compounds are cleared by ciliary movement or by uptake by macrophages in the lower airways after a certain time.

8 Discussion

Toxicity testing is performed to inform on chemical hazards or to assess human health risks from exposures during the stages of a chemical's life cycle. The regulatory information needs may vary from just a qualitative yes or no answer (e.g., does this chemical cause a specific effect) to a detailed evaluation of all possible health risks in a specific human exposure situation (Bos et al., 2020).

In vivo, toxicity is determined by the combination of the intensity, duration, and frequency of exposure, with dosing rate as an important factor for the toxicological outcome. Moving away from *in vivo* animal studies and using NAMs in human health risk assessment of inhalation exposure offers the advantage of varying the exposure conditions and the possibility to study a high number of parameters over time. This may provide important information on potential effects on the respiratory tract following inhalation exposure.

The development of *in vitro* methods for inhalation exposures is challenging because of their specific characteristics. The first topic is the design of the *in vitro* methods, in particular how aspects of exposure (intensity, duration, frequency) can be modeled so that test results can answer the regulatory questions of interest. A second topic is that an important goal of toxicity testing is the derivation of an exposure-response relationship, often referred to as a dose-response relationship, to provide information that may serve as a basis to assess human health risks caused by a specific exposure or to derive a limit value for human exposure. Where *in vivo* exposures focus on the relationship between health effects and external exposure characteristics, *in vitro* exposures focus on the direct contact of a chemical with cells or cell lines after exposure has ended (i.e., after deposition has ended). For modeling *in vivo* exposure to aerosols over hours, a time-dependent *in vitro* deposition may be too short. During and following exposure, clearance in the respiratory system will occur *in vivo*, which ensures that the chemical is no longer in contact with the cells. *In vitro*, depending on the system used, the deposited amount is well-controlled and is usually applied in a very short time period. Active clearance of insoluble particles in incubated cells is limited and can be substituted by apically washing the exposed cells. Otherwise, the particles will (i) stay on the mucus layer of the cells, (ii) be moved to the edge of the cell culture well, or (iii) be taken up by the cells.

Due to the differences in exposure and postexposure characteristics, it is difficult to directly relate the outcome of *in vitro* experiments to *in vivo* exposures in a quantitative way for the purpose of risk assessment or the derivation of a limit value for human exposure. Such an extrapolation must consider the *in vitro* exposure scenario, the biological effect, and their implications for *in vitro-in vivo* extrapolation and risk assessment.

8.1 Considerations for the *in vitro* exposure scenario

We have defined exposure duration (Tab. 1) for different systems, but there may be marked differences in exposure duration between chemicals. Volatiles (and gases) are only in contact with the cells for a short time period when using quasi-ALI exposure, whereas aerosols, mainly those of insoluble compounds, will stay on the cells or on the mucus or lining layer. *In vivo*, such insoluble compounds are cleared by mucociliary clearance or taken up by macrophages. Willoughby et al. (2015) used quasi-ALI exposure, did not wash the cells, and measured after a 24- or 72-h post-exposure period. This approach is comparable to submerged exposures for different durations. On the other hand, Zhang et al. (2020) washed the cells after a post-exposure period and repeated this to create a repeated exposure over multiple days. The latter seems more comparable to human inhalation exposure.

The literature does not yet describe *in vitro* studies that vary exposure concentration, duration, and frequency at the same total dose. The limited information available indicates that these factors can influence the test outcome. Therefore, to use *in vitro* toxicity information for human risk assessment, insight is needed on when and how variations in exposure concentration, duration and frequency determine toxicity. Although a study design varying these factors is quite labor-intensive, it will give information that is essential for risk assessment of inhaled toxicants. As an example, a study design in which the concentration, duration, and frequency is varied is shown in Table S3[2]. In addition, supplementary files 2[3] and 3[4] describe experimental designs in which the total dose or the dose rate was kept constant.

8.2 Considerations for the biological effect

Effects of exposure to higher concentrations may differ substantially from effects at lower concentrations. Information on the mechanism of action may help to identify biological processes or steps that are rate-limiting, like the saturation of receptors, to derive a relevant and adequate exposure-response relationship and to select suitable post-exposure periods for assessment of various toxicological parameters. The *in vitro* exposure scenario should be relevant (or at least be applicable with small adjustments) to possible human exposure scenarios, including high accidental exposures. A central role herein is foreseen for the AOP concept, i.e.,

to understand the perturbation of biological processes and to align testing strategies with that concept.

Cell models may respond differently to stressors dependent on other factors such as the time of day when they are exposed or the moment of seeding (Pamies et al., 2022). It is therefore important to treat negative control cultures the same as exposed cultures. Kooter et al. (2017) proposed a randomized exposure scheme, where among others the effects of exposure on different days, effects of different cell donors, and position in the exposure module can be accounted for. Insight into the sources of variation is important to be able to detect concentration-related effects.

8.3 Implications for *in vitro*-*in vivo* extrapolation and risk assessment

To be able to use *in vitro* data for risk assessment, it is important to relate *in vitro* dosing and cellular effects to *in vivo* human exposure and adverse outcomes. Information on biological effects and the impact of varying concentration, duration, and frequency in an *in vitro* experiment on the onset and progression of these effects is needed. Therefore, to use NAMs for human risk assessment, important topics to be considered are what kind of information is needed to answer a specific regulatory question, what kind of *in vitro* method will best provide that information, and how should the exposure in the *in vitro* test be to optimally model the *in vivo* exposure of interest.

For gases and vapors, *in vitro* exposures may mimic real-life exposure scenarios relatively easily. For aerosols, the only way at the moment to relate *in vitro* exposure to *in vivo* exposure is to link *in vitro* test results with *in vivo* exposure by aligning the deposited dose. To generate data that can be used for risk assessment, the following steps might then be taken: First, formulate the regulatory question to be answered and the information needed to answer that question. Starting from the human exposure scenario, estimate or model the deposited dose (e.g., using the MPPD model). Next, consider the *in vivo* health outcome of interest and verify how this can best be addressed by *in vitro* read-out parameters. The availability of an AOP would be helpful to choose the appropriate parameters. Based on these steps, an appropriate *in vitro* method can be chosen such that it can be extrapolated to the *in vivo* exposure. This includes the type of cells or cell lines (including their origin), the exposure design (starting from the *in vivo* deposition), the type and number of parameters to be measured, and the timing of the measurements. To extrapolate the *in vitro* test outcome to *in vivo* exposures, the *in vitro* deposition preferably should be measured. Finally, based on the deposited dose and the exposure design, the *in vitro* results may be extrapolated to an *in vivo* exposure (MaHock et al., 2021).

It is acknowledged that the previous paragraph describes an ideal situation, and although this is not commonly applied, there are some examples showing the feasibility of the use of *in vitro* models for risk assessment of inhaled substances (Ramanarayanan et al., 2022; Corley et al., 2021). Still, the current *in vitro* models lack predictivity for *in vivo* effects, which is important for the use of *in vitro* data for risk assessment. The development of AOPs for respiratory tract effects from chemical exposures is therefore essential. The use of different exposure scenarios in *in vitro* experiments, together with measurement of responses over time, will help to identify pros and cons of different *in vitro* models in their use for risk assessment. Keeping in mind what steps need to be taken to use *in vitro* test results for human hazard or risk assessment will help to identify the data gaps and to understand the limitations of the presently available methods. This will help to put tools in place and to develop them further for use in hazard or risk assessment.

8.4 Recommendations for *in vitro* studies to assess inhalation toxicity

In summary, the evaluation of the literature and our experience with different cell models and exposure systems has resulted in the following recommendations for *in vitro* experiments to assess toxicity of inhaled chemicals with the aim of supporting risk assessment:

1. Consider what information really is needed for hazard or risk assessment: What is the regulatory question to be answered or the regulatory need to be met?
2. Choose the exposure system that is suitable for the chemical or particle of interest, the appropriate exposure method to vary the exposure characteristics, and an exposure scenario relevant for the *in vivo* situation, including potential accidental high exposures.
3. Aim for a deposited dose that is relevant for human exposures at the specific site in the respiratory tract and consider washing the cells on the apical side after a predefined period to mimic mucociliary clearance when fully differentiated airway cultures are used.
4. Consider the post-exposure sampling times, whether the effect is initiated at the start of the exposure, measurement of response over time, and the side of the cell culture from which the samples are taken.

These recommendations can be used to improve *in vitro* study design in hazard and risk assessment on inhalation exposures and will be used as a guidance in our further projects including the NWA-ORC VHP4Safety project.

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Conflict of interest

All authors declare to have no conflicts of interest.

Data availability

Experimental data can be found in the supplementary files. For further information or questions, please contact the corresponding author.

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