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Cell Culture Techniques Essential for Toxicity Testing of Inhaled Materials and Nanomaterials *In Vitro*

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Introduction

Human tissue is bombarded by a huge range of chemicals. Our lungs are inhaling pollution from both stationary and mobile sources as well as inhaled nanoparticles (NPs) and therapeutic products designed to provide new and innovative medical solutions. Our challenge is to identify what exposures are putting us at risk and balance the risk against benefits that we may receive from these chemicals and new products [1-4]. Advances in *in vitro* cell culture technology may provide some of the answers.

Regulatory toxicologists and health and safety professionals need rapid and reliable information on hazard profiles of chemicals to be able to assess the risk and manage potential exposures to harmful materials. *In vivo* studies require a large number of expensive, time consuming and in some cases non humane tests in animal species [3]. Recent innovations in human cell culture exposure and test systems has allowed the development of *in vitro* assay systems that are predictive, representative and suitable for toxicity screening of a diverse range of chemicals including airborne materials and nanomaterials (NMs) [1,3,5]. Innovative *in vitro* exposure techniques that have been developed to provide direct exposure of human lung cells to inhaled materials and nanomaterials will be discussed. Further development and validation of such test systems are crucial for evidence based safety evaluation and risk assessment of inhaled materials, nanomaterials and therapeutic products.

Exposure to Inhaled Materials

Inhalation is the most common route of exposure for airborne materials and nanomaterials to enter our body either unintentionally or deliberately. The range of airborne materials may include individual airborne contaminants, diesel exhaust and complex atmospheres, aerosols of nanoparticles and nanomaterials and inhaled therapeutics.

Individual airborne contaminants

Exposure to airborne contaminants is a major contributor to human health problems. Inhalation of gases, vapours, and aerosols can cause a wide range of adverse health effects ranging from mild irritation to systemic diseases [2,4,6-11]. It is possible through conventional chemical analysis techniques to sample and analyse contaminated air but, such data provides neither direct measures of toxicity nor the precise mechanisms that derive toxic effects. Inhalation is considered the most important means by which humans are exposed to airborne chemicals, however, available *in vivo* toxicity data is mostly from oral and dermal exposures rather than inhalation exposure. This demonstrates the importance of exploring new alternative approaches to provide toxicity information on various ranges of airborne materials. Developing *in vitro* techniques that are comparable to *in vivo* environments during inhalation exposures are crucial to provide evidence for precise mechanisms by which inhaled materials induce toxic effects.

Diesel exhaust and complex atmospheres

In real world situations such as occupational environments or polluted cities, people are exposed to a variety of airborne chemicals simultaneously. For example, exposures to industrial solvents and volatile organic compounds usually involve a mixture of chemicals rather than a single compound [4]. Smoke inhalation from tobacco smoke [12] or fire combustion products [13] is another example of human exposure to multiple airborne toxicants consisting of both gaseous and particulate fractions. Motor vehicles such as diesel powered engines are main contributors to air pollution in urban environments and industrial settings such as mine industry [14]. Diesel exhaust emission is a major health concern due to its complex nature of gases, particulates and nanoparticles and mixture of these [15]. Often the toxic effects of multiple exposures assumed to be additive but, other possible and more severe synergistic or potentiation interactions of chemicals or gaseous and particulate components of complex atmospheres need to be considered [4]. Considering the multitude of airborne contaminants that usually occur in real life and workplace environments, development of appropriate toxicity test methods would be vital for comprehensive toxicity assessment of complex atmospheres, identifying the main toxicants causing adverse effects and understanding the potential mechanisms responsible for toxic actions.

Inhaled nanoparticles and nanomaterials

With the rapid development and commercialisation of nanotechnology based products the pattern of human exposure to particulates is about to change enormously. While nanoparticles and nanomaterials may enter into human body via inhalation, dermal, oral and injection either unintentionally or deliberately, inhalation exposure to nanoscale (1-100 nm) particles has become an emerging health concern. Research suggests that NPs may react very differently from the parent materials due to their unique physicochemical characteristics such as small size distribution, large surface area to mass ratio and surface characteristics [1,5,16,17]. As research and business both continue to invest increasingly on nanotech products, more research is also needed to assess the potential adverse effects of nanoparticles. Safety assessment should be considered as an integral part of nanomaterial design and manufacture to ensure the health and safety of workers, consumers as

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well as the environment. Currently, several research projects related to nanotoxicology and nanosafety are being conducted worldwide by international organisations such as the OECD (The Organisation for Economic Cooperation and Development), WHO (World Health Organisation) and US NIOSH (US National Institute of Occupational Safety and Health), that can facilitate international collaboration to understand potential risks of NPs and to protect human health from exposure to nanomaterials.

Inhaled therapeutics

Many inhaled therapeutics are delivered to the lung to elicit a local remedial effect, usually for asthma, COPD (chronic obstructive pulmonary disease) and other lung disorders [18]. Recently, anti-inflammatory and anti-coagulant inhalable dry powder products such as heparin and pentoxifylline have been introduced as an alternative administration for the treatment of smoke inhalation [19]. As inhalation facilitates the rapid access of materials into the blood stream it can also provide an excellent route of administration for systemic delivery of therapeutics such as protein and peptide containing products. However, when chronic treatment is required such as systemic delivery of insulin, human growth hormone and other proteins, extensive toxicology studies of inhaled therapeutics would be essential.

Inhalation technology is an important issue in both preclinical and clinical studies of respiratory products because of the technical requirements of aerosol generation and delivery devices [20]. For toxicity evaluation of inhaled therapeutics, both local and systemic responses must be assessed. Local effects can be assessed by specific inhalation toxicology methods and toxicokinetics models can be implemented to predict systemic effects. Therefore, for comparisons between pre-clinical and clinical exposures in relation to toxic end points and prediction of clinical dose, pharmacokinetics information would be very informative [21].

In Vivo Models of Toxicity Assessment

Traditionally, inhalation toxicology data have been generated using animal models. Inhalation toxicity tests are carried out on test animals to identify the median lethal concentration (LC_{50}) of airborne chemicals causing death as a toxic end point in 50% of exposed animals. Later maximum tolerable concentration (LC_0) has been introduced as a more ethically acceptable endpoint. The OECD has established a number of Test Guidelines (TGs) for acute, repeated dose and sub-chronic inhalation toxicity testing (e.g. TG 403, 412, 413) [22]. However, there are some difficulties unique to inhalation studies that have been identified [23].

To conduct adequate toxicology studies, it is important to quantify the delivered dose. Estimating the dose received by animals is one of the challenging issues of inhalation toxicology as several factors may influence the actual dose such as airborne concentration, duration of exposure and respiratory characteristics of the test animal, which modulate the deposition/absorption pattern of the airborne material. *In vivo* inhalation assessments include the determination of regional localisation and quantification of the deposited material in specific anatomical sub-regions of the respiratory tract, followed by macroscopic and microscopic tissue evaluation, target organ toxicity and estimation of the clearance rate [18]. Therefore, the selection of animal species for inhalation studies is a key factor, that may influence the outcome of *in vivo* studies and hence the extrapolated human adverse health effects.

Generation and characterisation of high volume test atmospheres and reproducible exposure conditions are more complicated and expensive compared to oral and dermal exposures. This process requires specialised equipment and techniques to generate, maintain and monitor test atmospheres. Inhalation exposure systems involve several efficient and precise sub-systems, including a conditioned air supply system, an appropriate gas or aerosol generator for the test material, a dilution and delivery system, flow monitoring system/s, exposure chamber, real-time monitoring or sampling and analytical system, and an exhaust/filter or scrubbing system [24].

In Vitro Models of Toxicity Assessment

In vitro toxicology test methods for toxicity testing of inhaled materials have been developed using animal or human based cellular systems and relevant biological endpoints. *In vitro* test methods have the potential to serve as the primary choice for toxicity screening of inhaled materials and nanomaterials as they are convenient, cost and time efficient and involve no ethical issues. Typical *in vitro* bioassays include cell viability (e.g. trypan blue dye exclusion), cell metabolism such as tetrazolium salts (MTT, MTS and XTT) and membrane leakage such as loss of lactate dehydrogenase (LDH) assays [5]. Biological responses and toxicity endpoints resulted from nanomaterial exposure that can be related to their physicochemical characteristics have been reviewed [1,4,18]. Damage to cell walls and plasma membrane, ROS (reactive oxygen species) generation and oxidative stress are among common mechanisms responsible for toxicity of NMs.

To overcome the challenges and limitations of *in vivo* inhalation studies, practical *in vitro* methods for toxicity testing of inhaled materials have been developed using human airway cells, lung cells or tissues and implementation of target specific end-points [25,26]. Although *in vitro* lung models do not represent the complexity of the whole respiratory system, many questions related to inhalation toxicology can be answered using physiologically relevant human lung cells and tissue models replicating the native cellular structure and tissue architecture of the respiratory system [5,24]. Models of the human lung are available for all regions of the respiratory tract, including nasal, trachea bronchial and pulmonary region. However, lung cell culture models should be selected in a way to represent the most important features of their corresponding region in the respiratory tract.

Considering the complex nature of the architecture of the lung, various *in vitro* models can be implemented representing the proximal and distal regions of the respiratory tract. Cells and cell lines of human airway (e.g. calu-3 human cell line) and alveolar epithelium (e.g. A549 human epithelial type II cell lines) have been frequently used in toxicity testing of inhaled materials and nanomaterials [27]. In addition, alveolar macrophages are among the first cellular systems responding to inhaled materials and can be obtained as primary cultures of pulmonary lavage or cell lines. Insoluble or low water soluble gases and vapours as well as ultra-fine particles and nanoparticles have a potential to access deeply into the distal lung in which, the pulmonary epithelium is composed of two distinct cell types of alveolar type I (AE1) and alveolar type II (AE2) serving diverse essential functions in the alveolar region. The development of distal lung tissue models is challenging due to the multicellular nature and complex structure of the alveoli but, recently promising approaches toward construction of 3D alveolar tissue models have been reported [27-30].

The development and implementation of engineered tissue equivalents of the lung need to be investigated further, similar to those models designed for other organs such as the skin [31]. Engineered cell culture scaffolds provide mechanical support for cells in the form of meshes, sponges and films consisting of a wide range of synthetic

materials or natural biomaterials [31]. Recently, a morphologically and functionally differentiated *in vitro* cellular model of the human airway epithelium (MucilAirTM, Epithelix) has been commercialised that can be maintained at the homeostatic state at least for one year [32].

In vitro test systems using cultured cells can be controlled precisely providing more reproducible toxicity data than *in vivo* models but requires higher standardisation. The main technical challenge of *in vitro* testing of inhaled materials is to mimic inhalation exposure in cultured cells or tissues. Therefore, an optimal *in vitro* exposure system for studying the cellular responses to inhaled materials needs to meet several requirements [3,33-36]. Such requirements include:

- Continuous direct exposure of target cells to unmodified airborne materials with no intervening layer of media,
- Providing a significant time course to study potential biological effects,
- Continual nourishment of cells during the exposure time, and
- Supporting other requirements of the cellular system such as appropriate levels of pH, humidity and temperature,
- Duplication of *in vivo* parameters without system-related toxicity, and
- Offering an uncomplicated exposure system with a reasonable cost.

To address the basic requirement of gas phase exposure of airborne materials several exposure methods have been developed. Different features of such exposure techniques have been discussed in terms of their relevance, advantages and limitations [3,5,33,34]. These methods mainly include exposure of cells under submerged conditions, intermittent exposure procedures and more recently direct exposure techniques at the air–liquid interface (ALI). The latest will be discussed as the most relevant method that can fulfil the above mentioned requirements.

Direct exposure at the air-liquid interface

Over recent years, there has been a significant development and breakthrough in toxicity testing of airborne materials *in vitro* [3,5,34-42]. Human cells were cultured on commercially available porous membranes permeable to culture media (Figure 1). Cells grown on porous membranes accommodated in exposure chamber systems were adapted for dynamic delivery and direct exposure of human cells to airborne materials providing a very close contact between target cells and test atmospheres similar to *in vivo* condition. Dynamic delivery of test atmospheres and direct exposure of human cells to airborne chemicals under ALI conditions were achieved using specifically constructed exposure chambers such as the CULTEX system [40], or horizontal diffusion chamber (Harvard Apparatus Inc., USA) systems [36].

Cellular responses to individual airborne chemicals such as NO_2, NH_3, O_3 and SO_2 [36-38,41] and complex mixtures such as diesel exhaust [14,42,43], cigarette smoke [44] and fire combustion products [13] have been studied using human lung cells grown on porous membranes. The dynamic direct exposure technique at the air–liquid interface offers a continuous contact between unmodified airborne chemicals and target cells and technically simulates *in vivo* inhalation exposure as closely as possible. Mobile exposure systems that can be operated independently from the cell culture incubator utilising heat water circulation, heat block or a heat cartridge, offer an advantage for an on-site toxicity investigation of airborne contaminants and complex atmospheres [14].

A commercially available perfusion chamber system (MINUCELL) was modified to allow the homogeneous deposition of fine and ultrafine aerosols into the membrane surface for *in vitro* exposure purposes [45]. Uniform particle deposition and a well-defined dose were achieved through this experimental setup [46]. For dynamic direct exposure of target cells to nanoscale aerosols at the air-liquid interface the Karlsruhe exposure system (Vitrocell, Germany) was also used [47,48]. To determine the accurate dose reaching the target cells, the membrane insert was replaced by a quartz crystal microbalance inside the exposure chamber and exposed to nano-aerosols to predict the deposited mass per area unit.

Technical improvements were also made to the CULTEX (Vitrocell, Germany) system utilising inlet tubes with a hyperboloid-shaped air distribution section made from Teflon or surface-treated stainless steel with up to 80% deposition efficiency for NPs [49]. Further, the radial flow system was designed for exposing of cells to micro and nanoscale particles using the computational fluid dynamics analysis to ensure reproducible and homogeneous deposition of particles [50]. More recently, a new computer-controlled air-liquid interface cultivation system was developed for generating 3D cultures of the airway epithelium [51]. *In vitro* exposure techniques that accomplish the essential requirements of cell culture systems for toxicity testing of inhaled materials are developing rapidly providing advanced tools for integrated toxicity assessment of diverse range of airborne materials and nanomaterials.

Conclusion and Perspective

Recent innovations in human cell culture and exposure techniques have allowed the development of *in vitro* assay systems that are predictive, representative and suitable for toxicity screening of a diverse range of chemicals including inhaled materials. Standardised

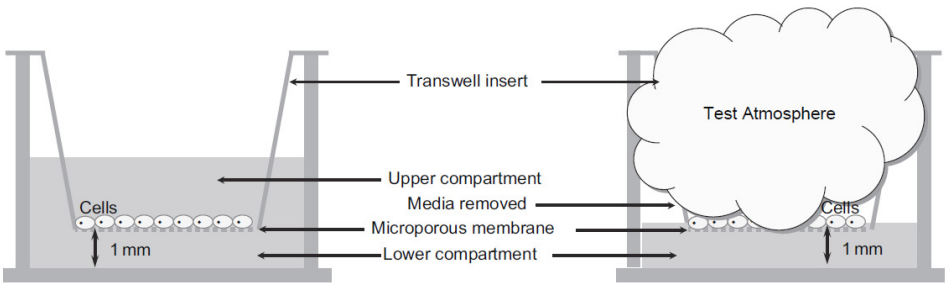

Figure 1: Direct exposure of human cells at the air-liquid interface.

in vitro test systems can be controlled precisely providing more reliable and reproducible toxicity data. An optimal *in vitro* exposure system for studying the cellular responses of inhaled materials needs to meet several technical requirements mainly the challenge of simulating inhalation exposure in cultured cells or tissues.

To overcome the challenge, innovative *in vitro* exposure techniques at the air liquid interface have been developed using human cells cultured on porous membranes that offer new possibilities to test biological responses of inhaled materials under physiologically relevant conditions. Such *in vitro* assay systems are an important research tools for developing a toxicity data bank on human exposure to a range of inhaled materials, nanomaterials and therapeutic products in a rapid reliable manner that will give regulatory toxicologists and health and safety professional's valuable evidence to assess the risk and manage potential exposures.

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