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REVIEW

IN VITRO CELLULAR MODELS, A RESOURCEFUL TOOL IN RESPIRATORY TOXICOLOGY

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Abstract

Due to the increasing concerns regarding the transferability of data from animal studies to potential human health effects, the development of complex *in vitro* cellular models which could potentially replace the ethically-debatable *in vivo* studies and fill the existing gap between *in vivo* and *in vitro* data has received considerable attention. The current review focuses on pulmonary *in vitro* cellular models available for studying the biological effects elicited by inhaled chemicals. The advantages and disadvantages of the 2D monoculture system and the more complex 3D models such as co-cultures and organ-on-a-chip platforms are discussed. Moreover, recent advancements in the field of respiratory toxicology such as the development of air-liquid interface systems that better mimic the *in vivo* respiratory exposure are reviewed. We conclude with future perspectives of the *in vitro* cellular models in respiratory toxicology.

Rezumat

Transpunerea datelor toxicologice rezultate din studiile pe animale în posibile efecte la nivelul sănătăţii umane este problematică, mai ales din cauza diferenţelor dintre specii. În acest context, a fost încurajată dezvoltarea unor modele celulare *in vitro* complexe care evită şi unele restricţii etice asociate cu studiile *in vivo*. Articolul de faţă se concentrează pe modelele celulare pulmonare *in vitro* disponibile pentru studierea efectelor biologice provocate de substanţele chimice inhalate. Sunt discutate avantajele şi dezavantajele sistemelor de tip monocultură 2D, precum şi ale modelelor mai complexe, 3D (co-culturi organotipice, platforme "*organ-on-a-chip*"). Sunt amintite de asemenea cele mai recente progrese din domeniul toxicologiei respiratorii, cum ar fi dezvoltarea sistemelor de interfaţă aer-lichid care imită mai bine expunerea respiratorie *in vivo*, precum şi perspective viitoare ale modelelor *in vitro* în toxicologia respiratorie.

Keywords: co-cultures, air-liquid interface, respiratory toxicology, lung

Introduction

Inhalation toxicology studies have been traditionally performed on animal models. However, difficulties regarding data transferability from *in vivo* studies into potential health impact on humans and the increasing concerns regarding the animal welfare have prompted the development of *in vitro* studies as alternatives [17]. *In vitro* model systems serve currently as a viable choice for toxicity screening of inhalable materials, as they are convenient, cost and time efficient. Although classical 2D monocultures of cells originating from specific regions of the respiratory tract do not reflect the complexity of the whole respiratory system, their usefulness in deciphering key aspects underlying physiological and patho-physiological processes in certain circumstances is undeniable. While for *in vivo* models it is almost impossible to improve their relevance because of the inherent differences between species, *in vitro* models using cell cultures are amendable for further improvements. In the last decades, complex *in vitro* models using differentiated primary cells, co-cultures of cells arranged in a 3D format on bio-engineered scaffolds and microfluidic systems have been developed, and hold the promise for a more thorough characterization of the potentially detrimental effects of inhalable compounds. Another important step for the improvement of *in vitro* models in respiratory toxicology was the development of exposure systems which allow the direct exposure to aerosols containing the inhalable material, thus closely mimicking native respiratory exposure. Moreover, in case of inhalable particles, the direct exposure of cells to the aerosol is even more important as the cellular uptake and subsequently their toxicity profiles can be considerably altered during conventional submerged exposure.

The aim of the current paper was to describe the possibilities available for studying the toxicity of inhalable toxicants using *in vitro* cell models. Advantages and limitations displayed by the current *in vitro* models are systematically discussed starting from the basic

2D monocultures and culminating with the state-of-the-art organ-on-a-chip platforms. A special interest was manifested towards describing the high relevance of 3D co-culture systems and other advancements in the field of respiratory as these improvements hold the promise to fill in the gap between *in vitro* and *in vivo* data.

Structure and function of the respiratory system

Inhalation is the main route of exposure to airborne toxicants, whereby the respiratory system serves as the primary port of entry and as a primary target. The respiratory epithelium forms a physical barrier with tight junction (TJ) complexes which is directly in contact with the external environment, offering protection to the underlying tissue. Depending on the section of the human airway, different types of cells including mucus producing goblet cells, ciliated cells and non-ciliated cells are organized into a protective pseudo-stratified epithelium [28]. In the alveolar region, two types of cells namely alveolar type I and II cells (AEC1 and AEC2, respectively) form the cellular basis for the gas exchange membrane. AEC1 cells cover up to 95% of the alveolar surface and due to their flatten morphology favour gas exchange processes, while AEC2 cells secrete the alveolar surfactant which prevents the alveolar collapse and can act as progenitor cells for AEC1 [18]. Besides this barrier function, epithelial cells are highly metabolic competent cells expressing an extended range of metabolic enzymes including the cytochrome P450 family and are able to metabolise inhaled toxicants [9]. At the tracheobronchial level, cilia and mucus act as a very efficient barrier for most of the inhaled toxicants, including particles by trapping and transporting them in the gastro-intestinal tract *via* the mucociliary escalator. In addition to the physical entrapment of foreign materials, mucus is also abundant in antibiotic factors such as antibiotic peptides and oxidizing enzymes which further act as defensive mechanisms [24]. In the more distal parts of the respiratory system cilia and mucus are not present, thus different humoral and cellular mechanisms of defence exist at the alveolar level. Humoral immunity is mainly mediated by pulmonary-surfactants A and D (SP-A, SP-D), lactoferrins, defensins and manose binding lectins, while cellular immunity is primarily mediated by alveolar macrophages (AM), recruited neutrophils and leukocytes [24]. AM play a key role in the initiation and the resolution of inflammatory processes, displaying a high plasticity and duality. Distinct micro-environmental cues can lead to differentiation of AM in subpopulations with antagonist effects such as cytotoxic/mitogenic, pro/anti-inflammatory, or fibrinogenic activities [1].

Nevertheless, the above mentioned defence mechanisms can be overwhelmed by the exogenous toxicants, and loss of barrier function caused by cellular death and local inflammation due to the recruitment of immune cells can occur. Moreover, in chronic diseases such as asthma and chronic-obstructive pulmonary disease (COPD), changes in the epithelial differentiation and the mucus production are observed [47, 68].

Required characteristics for cell culture models used in inhalation toxicology

The primary role of *in vitro* cellular models is to reproduce the function and organization of the native biological systems, in this case the respiratory epithelium, either in healthy or disease state. Moreover, an appropriate *in vitro* model should describe as closely as possible the mechanisms causing airway injury under exposure to inhalable toxicants, or the repair mechanisms in the case of inhalable therapeutic agents. The selection of the appropriate *in vitro* cellular model depends on the compound of interest and how it may interact with the exposed *in vivo* system.

An *in vitro* model used to assess the respiratory translocation of an inhalable toxicant or a therapeutic agent into the general circulation or other inner tissues should be able to develop a relevant physical barrier with a polarized epithelium to properly assess the toxicological or therapeutic implications. This is also required when the mechanism of toxicity is suspected to be related to the loss of barrier integrity. In addition, the ability of the *in vitro* models to secrete respiratory fluids such as mucus or alveolar surfactant is of crucial interest, as these secretions are present *in vivo* and have been found to mitigate the toxicity or lower the bioavailability of toxicants and therapeutic agents, respectively [34]. Besides these defensive mechanisms, the metabolic competence of the *in vitro* models should be comparable with the one observed *in vivo* as numerous inhalable agents are known to be detoxified or bio-activated by metabolizing enzymes in the respiratory system [9].

Even though 2D classical monocultures of cells do not fulfil the above mentioned requirements, further improvements were continuously made to encompass the previously experienced limitations. One major limitation encountered in the classical submerged cultures is the method of exposure to the tested compound [56]. This way of exposure is different from the native exposure of respiratory epithelia which occurs at the air-liquid interface (ALI), and may change the outcome of the experiments [42]. Various ALI exposure systems have been developed and are currently recognized as better alternatives to submerged exposure in respiratory toxicology [42]. Co-cultivation of cells originating from epithelial, immune or endothelial lineages has allowed a proper cell-to-cell communication, which underlies complex processes such as inflammation and sensitization [38]. Moreover, complex cellular 3D models cultivated on

bio-engineered scaffolds or inserts allowing a 3D orientation have emerged and may pave the way for a better transferability of data into potential health effects [36, 44, 49].

Types of epithelial cells used in respiratory toxicology

Primary epithelial cells

Primary human respiratory epithelial (nasal, tracheo-bronchial and alveolar) cells can be directly isolated from human tissue by (a)traumatic techniques. In addition, fully differentiated *in vitro* models of the tracheobronchial and alveolar regions are available from commercial providers (EpiAirway™ model from MatTek, MucilAir™ from Epithelix). The cultivation of isolated primary epithelial cells at the ALI on insert membranes leads to the development of a differentiated epithelium composed of both ciliated and mucus producing cells. For the propagation of cells, sub-merged conditions are well suited. By switching the cell culture to the ALI, the increased diffusion of oxygen is enhancing the cellular differentiation due to the accelerated oxidative cellular metabolism. Primary epithelial cells develop a tight physical barrier which is polarized and has a distinctive secretome in the apical and basolateral compartment when cultured on inserts [55]. Moreover, the metabolic activity of these differentiated models is similar to the one observed *in vivo*. When isolated from patients with chronic diseases such as asthma and COPD, primary cells maintain their disease phenotype, thus allowing experiments to be conducted on models for pathologic conditions [12, 39, 43]. This ability of primary epithelial cells to maintain their disease phenotype can be also useful for testing therapeutic agents for chronic respiratory diseases.

Even though cultured primary cells offer the closest resemblance to the native tissue in healthy and disease status, difficulties stemming from the isolation procedure and complex cultivation requirements, heterogeneity and high inter-donor variability and lastly their limited life span, hinder their use in toxicity assessment. Regarding the limited life span of isolated primary cells, recent advances in the field of conditionally reprogrammed cells have allowed the exponential growth of the isolated primary cells in the presence of a Rho-kinase inhibitor and of a feeder cellular layer without the need to alter the genome of the cells [8, 30].

Cell lines

Even though the continuous epithelial cell lines do not display the same capacity of differentiation as isolated primary cells, they have been used successfully in toxicological studies in certain circumstances. Cell lines originating from a single donor present a smaller heterogeneity than primary cells and are easier to cultivate. Moreover, due to their nature (immortalized or tumour origin) they have an extended life span, thus making large scale studies possible. Several cell lines representative for each section of the respiratory tract including nasal, bronchial and alveolar region are available and have been frequently used as models for the evaluation of airborne toxicants.

RPMI-2650 cell line (ATCC CCL30®) is the only available human nasal epithelium cell line at this moment. The cultivation at the ALI is the most critical requirement for reproducing *in vivo* like structures and properties [5, 40]. Similar to *in vivo* nasal epithelia, RPMI-2650 is able to form a polarized epithelium with similar transepithelial electric resistance (TEER) values and well-developed TJs [21, 40]. Moreover, RPMI-2650 cells can produce a mucoid material and show a similar metabolic activity as normal nasal epithelium cells [41, 53]. The major drawbacks of this cellular model arise from its low differentiation ability [64].

For tracheobronchial segment of the respiratory tract, three epithelial cell lines namely, Calu-3, BEAS-2B and 16HBE14o- are routinely used. BEAS-2B cells retain a typical epithelial polygonal appearance with a normal anti-oxidant capacity, and a similar expression of genes involved in the metabolism of xenobiotics as observed in primary bronchial epithelial cells [4, 35, 61]. They display a non-malignant phenotype, making them useful for studies of multistage bronchial epithelial carcinogenesis, with the p53 missense mutation having no impact on the functionality of this gene [51]. Similarly to other respiratory epithelial cell lines, the innate defence mechanisms represented by mucus secretion and mucus clearance are not present in BEAS-2B models as they do not display the differentiated epithelium observed *in vivo* [19, 66]. The existing data regarding the capacity of this cell line to develop a tight polarized epithelium in sub-merged and ALI conditions are sparse, with TEER values below 100 Ω/cm^2 and as high as 1000 Ω/cm^2 being reported [29, 61,69].

16HBE14o- is an immortalized tracheal epithelial cell line transformed with the SV40 large antigen. This cell line maintains a differentiated epithelial phenotype characterized by a polarized epithelium with well-developed surface microvilli and cilia [70]. 16HBE14o- cell line displays a similar response pattern as primary bronchial epithelial cells when challenged with various stimuli [2, 25]. Dissimilarities between 16HBE14o- and primary bronchial epithelial cells regarding the expression of genes involved in the xenobiotic metabolism were reported recently [14, 16]. 16HBE14o- cells express high levels of TJ proteins, and under submerged conditions they develop a tight epithelial barrier with TEER values higher than 500 Ω/cm^2 [45, 59].

Calu-3 is a well characterized cell line which forms a tight epithelial barrier with high TEER values, being

routinely used to model the respiratory epithelial barrier in toxicology or drug delivery studies [22]. Cultured at ALI, Calu-3 cell line develops a functional epithelium with microvilli and secretes a mucoid material which incorporates mucins such as MUC5AC. Moreover, when cultivated over longer periods of time, a rugged appearance denoting a pseudostratified epithelium was obtained [60].

For the alveolar region of the respiratory tract the most used cell lines are A549 and NCI-H441. Even though A549 was considered and routinely used as a continuous cell line representative for AEC2, some controversy regarding the phenotype of this cell line still exists. In a recent study it was reported that the development of an AEC2 phenotype is dependent on the long term culture of A549 in an appropriate medium [13]. By Raman Spectral Phenotyping, A549 cells were found to be more similar in nature to AEC1 than AEC2 [62]. In addition to the uncertainty regarding the utility of A549 cells as surrogates for AEC2, a major difference between cultured A549 cells and primary AECs is the inability of the former ones to establish an epithelial barrier with high TEER values [11]. Several components of TJs such as *zona-occludens*, claudins and occludins were detected at transcriptional and translational level, but the high permeability and the low TEER values are indicative of defective TJs [11].

NCI-H441 cell line presents a cuboidal morphology with microvilli and cytoplasmic structures characteristic for AEC2 cells [20, 26, 27]. The expression of surfactant proteins (A-D) and thyroid transcription factor 1 (TTF-1), typical markers of AEC cells, were also reported in NCI-H441 [27]. Similarly to the *in vivo* alveolar epithelial barrier, NCI-H441 is able to form a polarized epithelium with strong TJs and TEER values up to 1000 Ω/cm^2 [26, 50, 57]. Despite the apparent advantages offered by NCI-H441 over the A549 cell line, the use of this cell line in respiratory toxicology studies is limited due to the slower growth rate.

Induced pluripotent stem cells

The use of stem cells as progenitors for primary lung epithelial cells was described in literature and may encompass the limited lifespan of isolated primary lung epithelial cells. The induced pluripotent stem cells (iPSC) directly generated from somatic cells by gene editing the four Yamanaka factors (Oct4, Sox2, cMyc and Klf4) into the cell-genome offers a convenient alternative to the ethically-debatable embryonic stem cells [63]. Similarly to embryonic stem cells, iPSC can be guided to differentiate into specific cell types including respiratory epithelium cells. Moreover, the use of iPSC made the generation of patient specific *in vitro* cultures possible and is a breakthrough in the field of personalized medicine [63]. The use of iPSC can offer an unlimited supply of patient specific pluripotent cells starting from somatic cells isolated through non-invasive (urine) and minimally-invasive (blood) techniques. However, the differentiation process is expensive and needs specific factors at specific time intervals, thus limiting the use of iPSC in toxicological assessments.

Complex cellular models

Traditionally, the cells of choice for studying the effects of inhalable toxicants were represented by epithelial cells as these cells are directly exposed to inhaled compounds. However, in the structure of respiratory epithelium, several types of cells communicate and elaborate the biological response to the external stimuli. Even though *in vitro* cell cultures will never achieve the complexity of the whole respiratory system, further improvements in the complexity of *in vitro* models are necessary to study more intricate processes such as inflammation and respiratory sensitization [38]. It should be emphasized that a compromise regarding the complexity of the *in vitro* models needs to be done as one of the main advantages of these models is their simplified nature which allows to better control possible variables and to use high throughput techniques. For the development of such models, porous membranes (inserts), which allow the cultivation of cells on both sides, are generally used. By selecting from the various pore sizes available, different models which limit or on the contrary, favour the contact between the different cell lineages is possible and further allows studying the type of communication between the different cell types (paracrine or direct cell-to-cell contact).

Based on these considerations, co-cultures of epithelial cells with other cell types including immune cells (differentiated macrophages, mast and dendritic cells), fibroblasts and endothelial cells have been developed to investigate the key pathophysiological mechanisms of toxicity behind inhaled toxicants [3, 26, 38, 44]. The establishment of co-cultures is not a trivial task, as each type of cells needs its own type of cellular media and supplements. Moreover, the ratio between the different cell types needs to mirror the *in vivo* situation in disease or normal status. Nevertheless, the establishment of co-cultures is still feasible as plentiful solutions do exist [38].

Besides the physical barrier provided, epithelial cells play a central role in the initiation and the resolution of inflammation processes as these cells are involved in the recruitment of immune cells at the inflammation site. This is considered to rely not only on the release of soluble cytokines and mediators, but also on the direct cell-to-cell contact. Due to the permanent exposure to various stimuli, AM are less prone to induce a strong pro-inflammatory response which could be detrimental to the organism. This self-modulatory effect was also replicated during *in vitro* co-cultivation of epithelial cells (A549) with macrophages (THP-1)

when the LPS-induced pro-inflammatory mediators were decreased in the co-culture system [58]. Moreover, the communication between epithelial and immune cells is bidirectional as several studies showed that epithelial cells co-cultivated with immune cells develop a more differentiated epithelium with higher TEER values and more abundant apical secretions (mucus or surfactant) [15]. The inclusion of immune cells in co-cultures offers the possibility to obtain *in vitro* models representative for healthy and diseased respiratory tissue. This was recently described for the development of two different alveolar models in healthy and disease status by incorporating the M1 (pro-inflammatory) and M2 (anti-inflammatory) subtypes of macrophages in a co-culture of epithelial and endothelial cells [33].

The development of co-cultures offers in addition the possibility to evaluate indirect effects on the underlying types of cells, mimicking thus the *in vivo* situation. Endothelial cells which form a layer under the respiratory epithelium are key players in the initiation of the pro-inflammatory response as they favours the recruitment of immune cells from the systemic circulation at the inflammatory site [37]. As both epithelial and endothelial cells communicate in order to propagate or resolve inflammatory processes, both cellular layers can be considered as one integral functional unit [10]. Moreover, endothelial cells have been shown to influence the growth and differentiation of epithelial cell line under no other stimuli [10]. Co-culture of different endothelial and epithelial cells has been successfully used for the evaluation of inhalable toxicants, with the apical exposure of the epithelial side inducing an indirect response on the endothelial side propagating endothelial activation and dysfunction [6, 37, 54].

The current studies which evaluated the response of co-cultures to inhalable toxicants indicate that co-cultures better mimic the responses observed *in vivo* as the overall toxicity is lower, and the inflammation process is better preserved, most probably due to the presence of macrophages [3, 67]. Moreover, the genotoxic potential of particulate matter, inhalable particles resulting from combustion processes, has been shown to be lower in co-cultures, emphasizing the active role of macrophages in the defence against inhaled toxicants [32].

Even though commercially available porous membranes are accessible and convenient, in studies intended to evaluate the transport through the epithelial barrier, they can impede the transport of macromolecules or other physical entities such as nanoparticles or particles due to their thickness and pore size. To encompass this limitation, other kind of solid supports such as ultrathin porous silica membranes have been developed and can be used as an alternative [23]. Moreover, the use of inserts alone does not replicate the 3D architecture and the *milieu* formed by the extracellular matrix observed *in vivo*. Tissue engineered scaffolds based on polymers have emerged as a solution to the above mentioned limitation. If constructed properly, these scaffolds can incorporate in an architectural arrangement of extracellular matrix different cell populations, thus constructing an *in vitro* system similar to native micro-environments. An increased interest in using decellularized respiratory tissue of the tracheae or lungs for the development of more realistic 3D *in vitro* models was recently manifested [46, 48]. Moreover, shape-specific 3D scaffolds were constructed from solubilized decellularized tissue by 3D printing the soluble extracellular matrix components [52].

From the plentiful number of alternatives which exists and can be used to develop complex cellular *in vitro* models, the use of microfluidics coupled with co-cultivation of different cell lineages in a 3D format represents the state-of-the-art for *in vitro* models. The above mentioned features are reunited by the so called "organ-on-a-chip" platforms [7]. The first "lung-on-a-chip" platform, composed of human alveolar epithelial cells and human pulmonary endothelial cells cultivated on the two different sides of a flexible porous membrane was reported by Huh *et al* [31]. By incorporating the membrane in-between compartmentalized channels the flow of air and liquid in different compartments allowed the development of an air-liquid interface. Moreover, similarly to the stretch and relax motion present in the respiratory tissue, the developed model mimicked the breathing motion by applying positive and negative pressures in two side channels. Different effects were observed after a bacterial infection and nanoparticle exposure in the dynamic system when compared with the static system, further reiterating the importance of mimicking the physiological conditions in *in vitro* studies [31]. Similar "lung-on-a-chip" platforms were used to investigate the biological mechanisms behind pulmonary oedema, asthma, COPD and lung cancer. The interest in developing "organ-on-a-chip" has grown continuously since 2012 as they are considered to gradually replace the animal models and other *in vitro* studies which often lack predictability in drug discovery and testing, thus making drug design cheaper and the drugs more affordable to the population. Additionally, "organ-on-a-chip" platforms can be coupled in a modular way to create a "body-on-a-chip" platform which can include patient specific differentiated iPSC, holding the promise for the long-awaited personalized medicine [65].

Concluding remarks

The future of inhalation toxicology lies in the bench-marking of the currently existing *in vitro* models. Systematic testing in controlled conditions should pin-point the advantages of these models and ultimately their utility in the evaluation of inhaled toxicants. As currently there is a gap between *in vivo* and *in vitro*

toxicology data, more comparative studies between these complex models and animal models should be performed in order to validate the results obtained with the complex cellular models. Even though an *in vitro* model which would be able to respond to all questions of interests is still far from being achieved, further improvements of the current models should fill the gap between *in vivo* and *in vitro* studies and further allow cheaper, ethically acceptable and high throughput toxicity evaluation.

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