

REVIEW ARTICLE

Toxicological evaluation of airborne particulate matter. Are cell culture technologies ready to replace animal testing?

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Abstract

Exposure to atmospheric particulate matter (PM) can affect human health, causing asthma, atherosclerosis, renal disease and cancer. In the last few years, outdoor air pollution has increased globally, leading to a public health emergency. Epidemiological studies have reported a correlation between the development of severe respiratory and systemic diseases and exposure to PM. To evaluate the toxic effect of PM of different origins, conventional experimental toxicological investigations have been conducted in animals; however, animal experimentation poses major ethical issues and usually differs from human conditions. As an alternative, human cell cultures are increasingly being used to investigate cellular and molecular mechanisms of PM toxicity. Although 2D cell cultures have been proven helpful, they are far from being a valid alternative to animal tests. Recently, 3D cell culture and organ-on-chip technology have provided systems that are more complex and that can be more informative for toxicity studies. In this review, the results of the 2D systems that are most frequently used for PM toxicity evaluations are summarized with a special focus on their limitations. We also examined to which extent 3D cell culture and particularly the organ-on-chip technology may overcome these limitations and represent effective tools to improve airborne PM toxicity evaluations.

KEYWORDS

2D cell culture, 3D cell culture, airborne particulate matter toxicity, lung-on-chip

1 | INTRODUCTION

Air pollution is now recognized as a major environmental risk for respiratory and systemic diseases. According to data from over 3000 cities provided by the World Health Organization, outdoor air pollution has grown 8% globally in the last 5 years, with billions of people around the world now exposed to dangerous air (Vidal, 2016). Human populations in fast-growing cities in the Middle East, Southeast Asia and the Western Pacific Region in particular are exposed to pollution levels that are 5 to 10 times above World Health Organization-recommended levels of micrometre-size particulate matter (PM). This could be a public health emergency with huge future medical and social costs. For this reason, there is a global need to address growing environmental and clinical problems related to air pollution (Schulze et al., 2017).

PM is an important part of air pollution and is generally classified into different particle size groups: ultrafine (aerodynamic diameter $\leq 0.1 \mu\text{m}$), fine (aerodynamic diameter $\leq 2.5 \mu\text{m}$, PM_{2.5}) and coarse (aerodynamic diameter $\leq 10 \mu\text{m}$, PM₁₀). PM size determines how deep particles travel inside the bronchial tree and are deposited in the lung tissue. After inhalation, the fine and ultrafine fractions are able to reach pulmonary bronchioli and alveoli and interact with the epithelial barrier. There is evidence in the literature that ultrafine PM may also be found in the circulation and be dangerous for other organs such as the liver and kidney, as well for the cardiovascular system (Dey et al., 2015; E.-A. Kim, 2017; Terzano, Di Stefano, Conti, Graziani, & Petroianni, 2010; Sacks et al., 2011).

To identify the toxicity of different air pollutants for human populations, and to develop an environmental health policy, it is necessary

to estimate and monitor the toxic effects of exposure to PM from different sources. Over the years, the toxicity of PM has been investigated using various approaches (see Figure 1), in many cases through animal testing usually performed by incubation/inhalation in experimental animals (Stanek, Brown, Stanek, Gift, & Costa, 2011). Results from *in vivo* toxicological research showed that, after inhalation, PM is deposited in the lung, causing inflammation and stimulating the formation of reactive oxygen species (ROS) (Inoue et al., 2006; Nemmar, Al-Salam, Dhanasekaran, Sudhadevi, & Ali, 2009; Oberdörster, Oberdörster, & Oberdörster, 2005). Acute exposure to PM *in vivo* influences alveolar macrophage functions, such as cell migration and phagocytosis (Barlow, Brown, Donaldson, MacCallum, & Stone, 2008). In addition, several epidemiological studies found a correlation between brief exposure to PM and increased risk of respiratory morbidity, while chronic exposure to PM has been reported to induce cell hyperplasia and ultrastructural ciliary alterations of the airways epithelium in rats (Saldiva et al., 1992).

In spite of the useful results obtained through animal testing, there are important ethical and scientific issues in the use of experimental animals for these investigations. First, subjecting animals to increasing exposure times and concentrations of airborne PM to establish toxicity levels is considered unethical. In addition, another important limitation of animal studies is the extrapolation of findings from animals (usually rodents) to humans, due to species differences. In addition, animal studies are costly and time-consuming. For all these reasons, there is an urgent need to use alternative but reliable experimental strategies to predict PM toxicity in humans (Roggen, 2011).

The main alternative to animal testing is the use of human-derived cells, cultured with conventional *in vitro* techniques. The exposure of cells *in vitro* to airborne PM makes it possible to investigate interactions between microparticles and complex cellular biological reactions and pathways. However, despite these advantages, *in vitro* cell culture conditions poorly mimic the complex interaction of atmospheric airborne PM with living organisms, as they do not make it possible to reproduce functional cell-cell interactions nor to replicate organ-specific structural, mechanical and biochemical micro- and macroenvironments.

More recently, there has been a remarkable improvement in the capacity to culture human cells *in vitro* in systems that are more complex, such as co-culture conditions, 3D cell constructs, perfusion chambers and the so-called microfluidic-based organ-on-chip. A number of investigations have demonstrated that these systems enable the replication in the laboratory of specific cell and fluid interactions that recapitulate specific organ functions. Our aim was to review the literature to find out to what level these complex cell culture systems have been used at so far to address the difficult problem of studying PM toxicity *in vitro*, avoiding animal testing.

In this review, we examine the results that have been obtained so far regarding airborne PM toxicity using conventional cell culture techniques and innovative experimental techniques to mimic the complex structure and function of human cells more closely, mainly using cells from the lungs, such as co-culture systems, three-dimensional (3D) cell organization and the organ-on-chip technology. We discuss the importance and limitations of these approaches, aiming in particular to

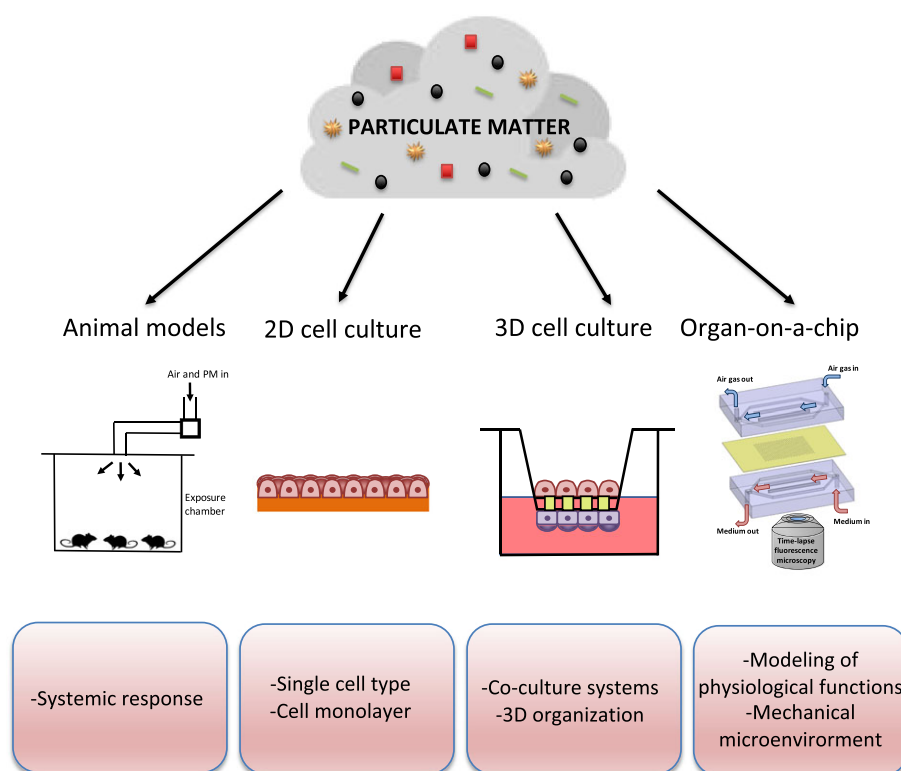


FIGURE 1 Available approaches for particulate toxicity investigations [Colour figure can be viewed at wileyonlinelibrary.com]

define to what extent the emerging organ-on-chip technology can address this important need in the toxicological field.

2 | OVERVIEW OF TWO-DIMENSIONAL IN VITRO SYSTEMS TO STUDY PARTICULATE MATTER TOXICITY

Studies related to PM toxicity in the respiratory tract are usually based on conventional two-dimensional (2D) cell culture systems. Monolayers of differentiated cells are usually cultured with conventional techniques and exposed to a given concentration of PM of different sizes and natures. Usually, PM toxicity experiments are conducted with cells grown and attached to the surface of polystyrene tissue culture dishes in static conditions (Jia, Wang, & Liu, 2017).

Reports in the literature of investigations into cell toxicity through in vitro PM exposure of mammalian cells are based on the use of different cell types. Thus, epithelial lung cell lines are commonly used to evaluate the effects of PM exposure. Lung adenocarcinoma cells (A549) and human bronchial epithelial cells (BEAS-2B/16HBE) are the most widely used human epithelial cell lines for toxicity studies (Don Porto Carero, Hoet, Verschaeve, Schoeters, & Nemery, 2001; Wu et al., 2017). The advantages of A549 cells are that they grow indefinitely and are easy to maintain in culture, while BEAS-2B cells are differentiated cells without genetic alterations.

Besides alveolar and bronchial epithelial cells, alveolar macrophages are also used to evaluate the effects of PM, as they play a major role in immune response, since these cells phagocytize micro-particles and cell debris, modulating the inflammatory response and maintenance of lung homeostasis (X. Y. Li, Gilmour, Donaldson, & MacNee, 1996; van Eeden, Yeung, Quinlan, & Hogg, 2005). Exposure of alveolar macrophages in vitro to PM_{2.5} has been shown to induce intracellular oxidation, apoptosis, phagocytosis and the release of pro-inflammatory cytokines (Luo et al., 2017; Miyata & van Eeden, 2011). Other types of cells have also been used in vitro for cytotoxicity tests, including lung fibroblasts derived from normal fetal lung tissue (MRC-5 and HFL-1 cell lines) (Jacobs, Jones, & Baille, 1970; Teramoto et al., 1997) and cells from embryonic lung tissue (L-132) (Abbas et al., 2010). As fibrotic alterations are crucial in airways remodeling (Jeffery, 2004), these cells are a good model for cytotoxicity studies (Abbas et al., 2010).

Transmission electron microscopy studies show that when pulmonary cells are exposed to PM₁₀ in vitro they interact with the cell surface and penetrate into the cell cytoplasm (Esposito et al., 2012). After PM exposure, the presence of multilamellar bodies increases (Esposito et al., 2012), indicating increased production of surfactant, and higher electron density of the multilamellar bodies is observed due to the presence of lysosomal enzymes. Further evidence of altered cell morphology after PM exposure came from Hong et al. (2016). These authors demonstrated, using scanning electron microscopy, that PM_{2.5} collected in the city of Shanghai adhered to the nasal epithelial cells and induced microvilli rearrangement. Similar effects were observed in A549 cells exposed to PM_{2.5} collected in the air in the city of Milan

(Gualtieri et al., 2009). PM was internalized in the cells through endocytosis after 6 and 24 hours of exposure. These findings are in line with those from Stearns et al. (Stearns, Paulauskis, & Godleski, 2001), who reported that after 24 hours of exposure, ultrafine PM was phagocytized by A549 cells, causing autophagy and cell necrosis.

In addition to cell morphology, exposure to PM in vitro affects cell viability (Peixoto, de Oliveira Galvão, & Batistuzzo de Medeiros, 2017), reducing cell proliferation and inducing cell death (Gualtieri et al., 2011). Oxidative stress is another important phenomenon that is usually induced during in vitro cell toxicity tests. A range of cellular responses (N. Li et al., 2002, 2003), including both protective and dangerous effects, depend on oxidative stress level. At low levels, the first response is the induction of antioxidant and protective enzymes. At higher PM concentrations, oxidative stress leads to cell death (N. Li et al., 2002; Nel, Diaz-Sanchez, & Li, 2001). The inflammatory response is characterized by the release of proinflammatory cytokines, chemokines and growth factors (Salvi & Holgate, 1999). Recent data show that fine PM can also be genotoxic agents (W. Kim et al., 2018), as oxidative stress may lead to DNA damage (Risom, Møller, & Loft, 2005), cell cycle mutation and, eventually, to carcinogenesis (Mordukhovich et al., 2010).

3 | LIMITATIONS OF TWO-DIMENSIONAL CELL CULTURE STUDIES

In spite of demonstrating important effects on cell structure and function, the evaluation of toxic effects of PM on conventional lung cell cultures in vitro has important limitations, due to the artificial culture conditions. Typically, in 2D cultures, cells are flattened and stretched (Breslin & O'Driscoll, 2013) and grow in rigid substrates in the presence of a single cell type. Moreover, cell monolayers are covered by culture media that allows the uniform distribution of nutrients and growth factors. Under these conditions, cell morphology is very different than under physiological conditions, with important differences in cell functions, such as protein and gene expression, proliferation and cell differentiation (Edmondson, Broglie, Adcock, & Yang, 2014). In the literature, there are a lot of studies demonstrating that gene and protein expression profiles for various cell lines grown in 2D or 3D culture are different (Gurski, Petrelli, Jia, & Farach-Carson, 2010; Lee et al., 2013). In particular, recent research has focused on A549 cell protein expression in 3D cultures, showing a lower quantity of protein expression in the former condition (Ravi, Kaviya, & Paramesh, 2016). Furthermore, cell function is importantly related to the cross-talk between differentiated cell phenotype in physiological conditions, while conventional 2D culture systems are usually based on a single cell type.

In physiological conditions, cells are mechanically coupled to their extracellular environment and mechanical inputs, due to the elasticity of the extracellular matrix, can regulate cell growth, proliferation, differentiation and activation of transcriptional regulators in different ways. The A549 cells exhibited different behavior, depending on cell substrate stiffness (J. Li, Han, & Zhao, 2014). When cells were cultured

on polyacrylamide, a positive correlation between spreading speed and stiffness of the substrate was found. Zhao et al. (2017) showed that on the stiffer polydimethylsiloxane substrates, A549 cells increased the production of matrix metalloproteinases and angiogenesis-related growth factors. In 2014, Dysart and co-workers (Dysart, Galvis, Russell, & Barker, 2014) demonstrated that the exposure to PM of A549 cells, cultured on substrates with increasing stiffness, increased the transforming- β signaling and mechanoactivation of these cells. All these observations underline the importance of the mechanical environment in cell toxicity evaluations and suggest that the results of conventional 2D cell culture systems may not be reliable.

2D culture systems exhibit another important limitation. Usually, epithelial pulmonary cells are exposed *in vitro* to PM under submerged condition. A more realistic method to simulate *in vivo* conditions more closely is to use cells exposed to the air/liquid interface (ALI). These cells can be cultured on a porous membrane in direct contact with the air. Exposure to silica nanoparticles has been shown to induce more significant effects under submerged conditions compared to ALI (Panas et al., 2014). Lenz et al. (2009) observed a different dose-response of mRNA expression of proinflammatory (interleukin [IL]-8) and oxidative stress (HO-1) markers after ZnO nanoparticle exposure under submerged or ALI cell exposure. Other investigations demonstrate that ALI is more appropriate for toxicological studies (Bitterle et al., 2006; Holder, Lucas, Goth-Goldstein, & Koshland, 2008).

4 | STRATEGIES USED TO OVERCOME TWO-DIMENSIONAL CELL CULTURE LIMITATIONS

To overcome the limitations of traditional 2D culture systems, new technologies have been developed. For instance, 2D co-culture systems, where two or more cell types are simultaneously cultured, can be used to elucidate the effects of cell-cell contact and/or secreted factor (Bogdanowicz & Lu, 2013). Co-culture models using various cellular associations were used to study PM toxicity. In 2008, a novel *in vitro* co-culture system was developed by Alfaro-Moreno and co-workers (Alfaro-Moreno et al., 2008) to study cytokine secretion after PM exposure compared to single cell culture. The study showed that cytokine secretion could be amplified or mitigated in co-cultures, depending on the type of cells used. Specifically, this study revealed that an amplified response to PM occurs when mast cells and macrophages interact. These effects mimic what happens in the lung, particularly to allergic subjects. In addition, tricultures of pneumocytes, macrophages and mast cells, exhibited a remarkable rise in cytokine release (Alfaro-Moreno et al., 2008). Wottrich et al. (Wottrich, Diabaté, & Krug, 2004) reported that the cells in the co-culture system after exposure to PM enhanced the production of cytokines compared to monoculture. Cultures of epithelial and endothelial pulmonary cells on transwell filter membranes show that the exposure of epithelial cells to PM induces a remarkable increase in IL-1 β release from the basolateral compartment, while no interleukin is released from the apical compartment (Bengalli, Mantecca, Camatini, & Gualtieri, 2013).

Using the same system, epithelial cells at the apical side of the co-culture membrane exposed to PM increased the release of interleukins by endothelial cells (Hermanns et al., 2009; Kasper et al., 2011). More recently, Klein and co-workers (Klein, Serchi, Hoffmann, Blömeke, & Gutleb, 2013), cultured endothelial cells and A549 cells seeded on to the two sides of a transwell with macrophage and mast cells. They showed lower ROS production and a lower release of IL-8 with this 3D culture system after cell exposure to PM, compared with monocultures of endothelial, macrophage and mast cells.

In 2016, Ferraz and co-workers (Ferraz, Rainho, Fernandes, & Felzenszwalb, 2016) developed a 3D hydrogel framework based on the alginate encapsulation of A549 cells. In this study, A549 cells cultured in 3D were less sensitive compared to monolayer cells. Indeed, a 3D tissue-like architecture was proven to help type II epithelial cells in the maintenance of alveolar function, as well as in preserving surfactant synthesis *in vitro* (Nandkumar, Ashna, Thomas, & Nair, 2015).

Although toxicity tests based on 3D cell culture systems are more closely related to native lung tissues than conventional 2D single cell monolayers, and thus more informative and reliable, they are based on static culture conditions. This condition limits nutrient transport and metabolic waste removal to diffusivity, while this transport in physiological conditions is due to convectivity. In addition, the absence of a dynamic culture fails to reconstitute mechanical features of whole living organs, such as hydrostatic pressure and fluid shear stress, which are central to their function (Huh et al., 2010). Thus, more sophisticated cell culture systems are needed to replicate the native tissue structure and function.

5 | CAN AN ORGAN-ON-CHIP ADVANCE PARTICLE MATTER TOXICITY TESTS?

One way to overcome the limitations of 2D and 3D *in vitro* cell culture systems is the use of microfluidic devices (see Table 1). In the last decade, major effort has been devoted to developing the so-called

TABLE 1 Key points to obtain an alternative to animal model for toxicological evaluation

	2D culture	3D culture	Organ-on-chip
Uniform nutrient transport and metabolic waste removal	No	No	Yes
Long-term culture	No	Yes	Yes
Air/liquid interface culture	No	Yes	Yes
Physiological particulate matter exposure	No	No	Yes
Possibility to include immune/circulating cells	No	Yes	Yes
Mimics aspects of human lung morphology and tissue organization	No	Yes	Yes
Mimics aspects of human lung physiology	No	No	Yes

organ-on-chip technology to recapitulate, in vitro, complex cellular functions of tissues and organs. These devices are designed to culture different living cells under perfusion conditions in micrometre size chambers that model cell and fluid interaction of physiological cell functions. This setting makes it possible to recreate functional cell-cell interactions and organ-specific mechanical microenvironments, including fluid shear stress, mechanical loading and cyclic strain (Huh et al., 2010). Such improvements make an organ-on-chip a central candidate for investigating the toxic effects of PM, and for studying the cellular pathway triggered by PM exposure.

The lung-on-chip microdevice was first reported by Huh and co-workers and was used to reconstruct the alveolar-capillary barrier (Huh et al., 2010). This microfluidic system has two closely opposed microchannels that are separated by a thin porous elastomeric membrane (10 μm thickness). Human alveolar epithelial cells are cultured on one side of the membrane, and human pulmonary microvascular endothelial cells on the other side. The device is integrated with two lateral microchambers to induce pressure-driven membrane stretching, which is present during normal inspiration. The presence of independent microchannels makes it possible to change fluid flow on cells, as well as enabling the delivery of nutrients to the epithelial and endothelial layer. The microdevice was tested in a nanotoxicology study that aimed to investigate the pulmonary response to silica nanoparticles that simulated air pollutants. The delivery of nanoparticles to the epithelial compartment induced the endothelial expression of the proinflammatory adhesion molecule, ICAM-1. Moreover, the measure of the intracellular production of ROS increased only when the alveolar epithelium underwent physiological breathing, showing that physiological breathing promotes acute toxic responses to environmental PM (Esch, Bahinski, & Huh, 2015). The role of the mechanical strain introduced in the first lung-on-a-chip was confirmed by Stucki and co-workers (A. O. Stucki et al., 2015), who designed a lung-on-a-chip with an integrated bioinspired respiration mechanism. Starting with the in vivo situation, where the diaphragm is responsible for the breathing process, they integrated a micro-diaphragm to stretch the alveolar-capillary barrier. The cyclic stretch in this system has a marked effect on cytokine expression and on the metabolic activity of alveolar epithelial cells.

Research and development in the area of lung-on-chip technology has recently been applied to drug screening (Benam et al., 2016; Skardal et al., 2017) and to the study of lung physiology (Benam et al., 2017; Henry et al., 2017; Humayun, Chow, & Young, 2018; J. D. Stucki et al., 2018) and pathophysiology (Huh et al., 2012; Jain et al., 2018), and we wonder whether this technology was also used in investigations related to environmental toxicology. However, although the technology is promising and does not have ethical and regulatory limitations, its use to study the response of human cells to ambient toxicants has not yet been presented in the literature (Cho & Yoon, 2017). This is surprising as this technology is a reliable alternative for toxicological animal studies with the potential to be easily scaled for higher-throughput screening, and despite the limited cost and equipment required for these investigations. At the moment the model still needs to be better

characterized and standardized but, despite the early stage of development, the use of the lung-on-chip technology is rather promising and can potentially overcome the major limitations of animal toxicological studies, particularly those related to excessive costs and ethical considerations. Importantly, this experimental approach can be used to perform toxicity tests based on cell viability and permeability assays, protein and gene expression analysis, immunostaining and ultrastructural investigations using electron microscopy. In addition, this experimental approach can be used to assess genotoxicity of PM (Nemmar, Holme, Rosas, Schwarze, & Alfaro-Moreno, 2013). All these observations can potentially identify the most toxic PM present in the environment, and to identify the chemical compositions that trigger the most dangerous biological response. In fact, it will be impossible in the future to reduce or eliminate airborne PM, but it will be necessary to identify the most dangerous airborne particles and concentrate efforts to reduce these components before it is too late to prevent it from having toxic effects on the populations of large urban and industrial environments.

6 | CONCLUSIONS

Currently, 2D cell culture systems still play an important role in basic toxicology research. Despite the limitations described above, 2D models are widely used to study the effects of PM due to fact they are easy to use and routinely applicable. This type of system may help to clarify the mechanisms responsible for PM toxicity, once the particles are inhaled, accumulate on the lung surface and interact with the pulmonary cells, inducing cell damage and eventually particle transport into the blood circulation. Although the 2D cell culture systems exhibit several limitations, they can be used to screen a large number of toxicants within a short time. The development of new, more accurate and high-performance in vitro systems to reproduce better the in vivo environment of cells and tissues is gaining importance for PM cell-based assays.

This aim can be achieved by a 3D system, as well as new cell growth substrate and dynamic cultures. Current data suggest that 3D systems influence cell behavior, differentiation and function. In addition, a dynamic cell culture creates a mechanical microenvironment that has important biological effects at the nuclear and protein level. Recently developed organ-on-chip technology is an important and crucial step to advance in vitro investigations on PM toxicity on pulmonary cells and other cell types of different organs (i.e., liver, kidney, etc.). These systems recapitulate the physiological and mechanical microenvironments of a living epithelial/endothelial membrane, allowing researchers to study the effects of airborne PM on human cell biology. This experimental strategy has great potential but has not yet been done previously. It is time to invest in developing the organ-on-chip technology as an efficient and reliable alternative to in vivo toxicological studies. This approach may allow researchers to improve the identification of dangerous airborne microparticles present in the urban and industrial environments.

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CONFLICTS OF INTEREST

The authors have no conflicts of interest to report.

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