 doi:10.20944/preprints202105.0450.v1

Review

Replacement strategies for animal studies in inhalation testing

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Abstract: Testing in animals is mandatory in drug testing and the gold standard for evaluation of toxicity. This situation is expected to change in the future because the 3Rs principle, which stands for replacement, reduction and refinement of the use of animals in science, is reinforced by many countries. On the other hand, technologies for alternatives to animals experiments have increased. The necessity to develop and use of alternatives is influenced by the complexity of the research topic and also by the fact, to which extent the currently used animal models can mimic human physiology and/or exposure. Rodent lung morphology and physiology differs markedly for that of humans and inhalation exposure of the animals are challenging. *In vitro* and *in silico* methods can assess important aspects of the *in vivo* action, namely particle deposition, dissolution, action at and permeation across the respiratory barrier and pharmacokinetics. Out of the numerous homemade *in vitro* and *in silico* models some are available commercially or open access. This review discusses limitations of animal models and exposure systems and proposes a panel of *in vitro* and *in silico* techniques that, in the future, may replace animal experimentation in inhalation testing.

Keywords: 3Rs; replacement of animals; inhalation; *in vitro*; animal models; species differences; lung morphology; rodents; aerosol exposure

1. Introduction

Animals are used in science worldwide but actual numbers are often unknown and difficult to compare between countries because the reporting varies considerably[1]. For example, list EU countries animal experiments, while most other countries count the number of animals used. Further, not all purposes involving animals are included. The U.S. counts only warm-blooded animals in research, teaching, and testing, not rats, mice, and birds that were bred for research. According to the most recent report of the European Commission more than 60% of the animals used in 2017 were mice, 12% were rats, 13% were fish and 6% were birds. Dogs, cats and non-human primates made up just 0.3% of the total. Animals are mainly used in basic science (45%), translational research (23%) and regulatory testing (23%)[2]. Many studies are linked to the development of medical products. The most recent analysis for bringing a molecule to the market indicated costs ranging from $765.9 million for therapies related to the nervous system to $2.7 billion for antineoplastic and immunomodulating agents [1]. A study published a decade ago, reported average costs of 1.5 billion for a marketed drug [2]. The time of drug development until approval was estimated as 10-15 years, with pre-clinical testing lasting around 5 years. The most expensive and longest part in the development are the clinical phases of the testing and it is, therefore, important that the development of not promising drugs is stopped in the preclinical phase. This phase includes target identification and dose finding in cellular screening, pharmacokinetic profile, pharmacodynamic profile, bioavailability

[1] https://speakingofresearch.com/facts/animal-research-statistics/

[2] https://www.nature.com/articles/d41586-020-00352-6

 doi:10.20944/preprints202105.0450.v1

and safety studies (acute and chronic toxicity testing, reproductive toxicity and teratogenicity, mutagenicity and carcinogenicity, immunotoxicity, local tolerance) in animals.

2. Animal testing and 3Rs

The opinion on animal testing in science and research varied in history [3]. Safety testing of drugs was requested by the Federal Food, Drug, and Cosmetic Act in 1938 after the tragic incident by 'Elixir Sulfanilamide'. The raspberry flavoring dissolved in diethylene glycol (DEG) should make the drug more attractive to the users and had not been tested in animals. The product caused the death of more than hundred people. Another drug fiasco, the "thalidomide scandal" supported the request for more extensive animal testing in the production of safe drugs [4]. Thalidomide was marketed under the name Contergan in the late 1950s and early 1960s and prescribed as medication for anxiety, sleep problems, and morning sickness. The poorly water-soluble compound showed poor oral absorption in animal studies and low acute toxicity. When taken by pregnant women between the 20th and 40th day after conception, severe malformations of the newborns were observed. As a reaction to this tragedy, reproductive toxicity testing and teratogenicity testing was mandatory for systemically acting drugs.

At about the same time, the scientists Russell and Burk introduced in 1959 the concept of the 3Rs, which stand for replacement, refinement and reduction of the use of animals in science. The principles defined by Russell and Burch were not completely clear to all researchers and over many years no fundamental changes in the use of animals in science was noted. The two researchers did not find the use of animals in research problematic, but the infliction of unnecessary or avoidable pain, fear, stress, anxiety, bodily discomfort and other significantly unpleasant feelings [5]. Marshal Hall promoted this idea many years earlier in his essay "on experiments in physiology as a question of medical ethics" published in 1847. Important milestones for a better implementation of the 3Rs came from the regulatory side by the provision of guidelines for *in vitro* characterization, mandatory approval of animal studies, implementation of Ethic committees, the European Centre for the Validation of Alternative Methods (ECVAM; Europe) and the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM; USA). From the scientific side the availability of more representative cell-based and computer models, disease-specific animal models, new imaging modalities, better experimental designs, improved statistic programs, data and tissues sharing, new biomarkers, anesthesia and analgesia management, and better analytical methods supported the 3Rs principle [6].

The above mentioned actions did not result in a rapid decline of the use of animals in all fields of research. The use of animals was reduced in safety testing of cosmetics because the Cosmetics Regulation released by the European Commission in 2009 prohibited marketing of products and ingredients in the European Union that have been tested on animals. However, a comparison of the use of alternative methods in the dossiers submitted to the Scientific Committee on Consumer Safety (SCCS) between the periods 2008-2013 and 2013-2016 showed that the increase in *in vitro* testing was not as pronounced as expected [7]. The authors hypothesized that this was due to the fact that several ingredients were developed before the ban. In contrast to regulatory testing of cosmetics, the study of various systemic diseases and their treatment will not be possible without animal testing.

Testing of inhaled substances, toxins or drugs, represents a field of research where alternative techniques may be more predictive than data generated in rodents. Acute inhalation toxicity testing in rats was not relevant for humans according to assessment of 52 studies, indicating that the currently used testing may not be optimal for inhaled toxicants [8]. The high rate of drug failure in clinical phases of 60% between 2011-2012 is an indication that animal models particularly in respiratory research are so poorly predictive for

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the human condition [9]. Further, there are only few animal models that show the relevant aspects of human respiratory diseases. Another limitation is the fact that animal procedures often involve sedation of the animal. Depression of respiration can lead to hypercapnia, hypoxia, acidosis and may impact cytokine secretion [10]. Such effects could be incorrectly interpreted as drug-induced effects.

This review highlights limitations in animal testing regarding specific-specific differences in anatomy, physiology and pathology of the respiratory system of the commonly used rodents and discusses the status of *in vitro* and *in silico* techniques as alternatives for efficacy and toxicity testing of drugs for oral inhalation and inhaled toxicants.

3. Respiratory diseases

Chronic respiratory diseases are relatively common and were identified as the third leading cause of death worldwide in 2017 [11]. Their incidence has increased from 1990 to 2017 by 39.8% and chronic obstructive pulmonary disease (COPD) (3.9% global prevalence) and asthma (3.6%) were the most prevalent diseases. In addition to the chronic respiratory diseases, acute lower respiratory tract infections not only predispose to chronic respiratory diseases later in life but also is also responsible for annually millions of deaths[3]. The pandemics caused by the severe acute respiratory syndrome coronary virus 2 (SARS-CoV-2) raised the awareness for the dramatic consequences of respiratory infections. Research and drug development focuses on COPD, asthma, cystic fibrosis (CF), pulmonary infections and pulmonary fibrosis as the most prevalent respiratory diseases.

3.1. Cellular screening

Identification of lead compounds in respiratory research starts with applying a series of attributes (potency, low oral bioavailability, rapid onset of action, low dose, high plasma protein binding and metabolic vulnerability) to design specific drug molecules [12]. In this process quantitative structure-activity relationship (QSAR) is often applied. Cell lines and specific (e.g. genetically modified) cells cultured on plastic surfaces and exposed to medium form the apical side (so-called conventional culture) are used in this step. Basal cytotoxicity can be performed in the same way. When a formulated product has been generated, a set of established particle characteristics, most prominently identification of the fine particle fraction (FPF), and dissolution are determined by *in vitro* techniques (Fig. 1).

[3] https://www.who.int/gard/publications/The_Global_Impact_of_Respiratory_Disease.pdf

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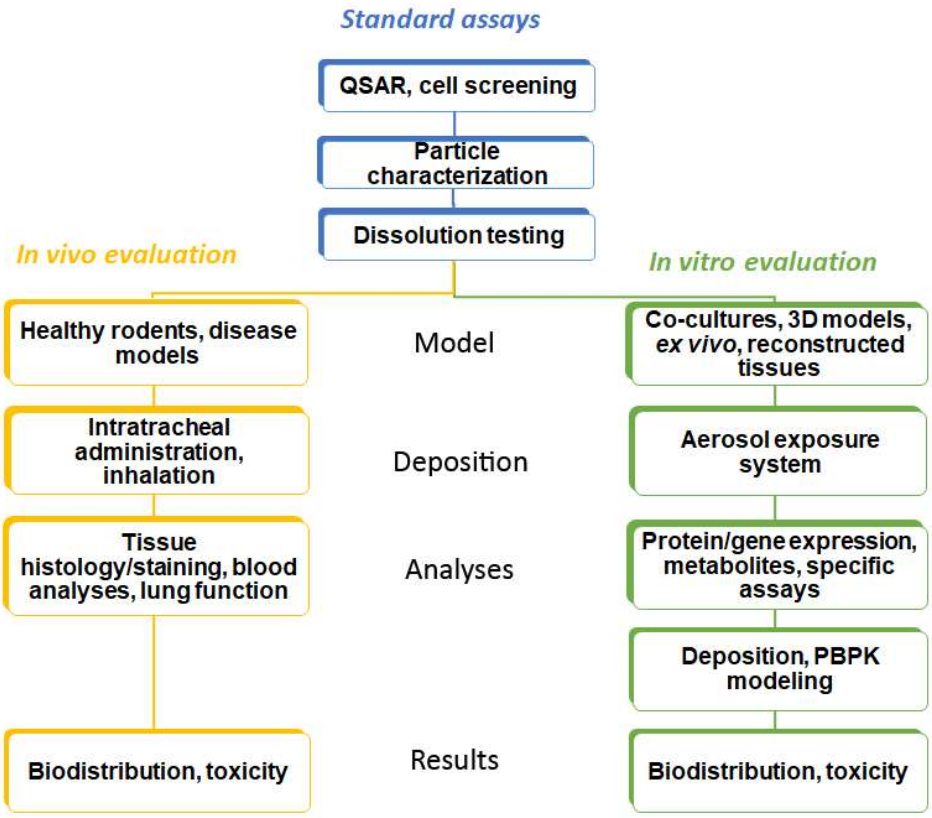

Figure 1. Workflow in the testing of inhaled substances. Common techniques in the testing are shown in blue, *in vivo* testing methods in yellow and alternative testing methods (*in silico* and cellular models) in green boxes.

3.2. Characterization of aerosols

Deposition is a very relevant parameter in the study of inhaled substances because it correlates directly with treatment efficacy and data are useful to show bioequivalence between different inhalers or formulations. Deposition pattern have been studied over the last 50 years and particle properties (size, shape, density charge, hygroscopy of particles) and airway geometry (gender, age, disease status) and breathing pattern (frequency, tidal volume, breath-holding) were identified as the most relevant parameters [13]. Mechanisms of particle deposition include sedimentation, impaction, diffusion, electrostatic effects and interception and are described in several reviews (e.g. [14]).

Established techniques for characterization of aerosols are described elsewhere (e.g. [15]). The FPF representing particles in aerodynamic size <5 µm) is regarded as most important parameter for deposition of orally inhaled drugs in the deep lung. It is correlated to lung deposition *in vivo* according to gamma scintigraphy. If different instruments (e.g. Anderson Cascade Impactor, Marple-Miller Impactor, Next Generation Impactor, Twin-stage Impinger, Multistage Liquid Impinger) are used FPF, differ because they work at different airflows [13].

3.3. Dissolution

Another relevant parameter for action in the lung is dissolution. Dissolution testing is well standardized for oral formulations and also for metal nanoparticles, where dissolution in the acidic environment of the lysosomes plays an important role. For inhaled drugs, there is a variety of different compositions, pHs and volumes of the

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dissolution fluid published in the literature. The different protocols will not be discussed in this review and the reader is referred to reviews dedicated to lung-specific dissolution [16,17].

4. *In vivo* testing in pulmonary research

This testing needs relevant animal models and aerosol exposure and a broad spectrum of analyses to determine biodistribution, efficacy and toxicity.

4.1. Respiratory system of healthy animals

Despite a general similarity of architecture and function of mammalian lungs, there are several morphological differences between human and rodent lungs. Similar to humans, rodent lungs have five lobes but the distribution between left and right lung differs. There is one (rodents) compared to two (humans) lobes in the left lung and 4 (rodents) and 3 (humans) in the right lung. Rodents have monopodial branching of the airways, while humans have symmetric branching pattern. The consequence of the symmetric branching is that deposition of particles occurs primarily at branching points. The lung parenchyma contributes to 18% in mouse, to 24% in rats and to 12% in humans of the entire lung volume [18]. Obviously, the length of bronchial tree, the diameter of the airways and of the alveoles are much smaller in rodents. The relative lumen of the airways, however, is larger in rodents than in humans, which results in lower flow resistance providing the physiological basis for the high respiration rate (250-350 bpm). Inflammatory processes compromise rodent less than the human lung function because the airways are larger and mucus glands are absent. However, due to the smaller absolute airway diameter, administration of larger amounts of particles may cause airway obstruction and induce death after instillation in rodents. The architecture of junctions between distal conducting airways and alveolar parenchyma is also different [19]. While terminal bronchioles and alveoli are connected by respiratory bronchioles in primates and carnivore lungs, rodents, equids, pigs and ruminants have (the thinner) alveolar ducts between these structures. The difference may explain why the proximal acinus in humans is more sensitive to smoke than the acinus of rodents. The rodent intralobular airways consist of simple epithelium without basal cells and the non-ciliated cells serve as progenitor cells, while humans have basal cells for regeneration [10]. The air-blood barrier is thinner in mice (0.32 μm) and rats (0.38 μm) than in humans (0.62 μm), which allows more rapid diffusion of gases and hydrophobic molecules in rodents. Furthermore, 50% of the human air-blood barrier surface is insufficient for diffusion because the barrier is too thick [20]. Collagen type I and occasional fibroblast hinder the exchange but are, on the other hand, necessary for mechanical stability of the delicate alveolar walls. Despite the mechanical support, intercellular epithelial junctions may break under physiological stress (exercise close to the maximal oxygen consumption in cyclists, mechanical ventilation of patients on the intensive care unit or living in high altitude) and lead to bleeding into the alveoles. Differences exist further regarding the absence of smooth muscle cells beyond the bronchoalveolar duct junction, smaller size of alveolar macrophages and the presence of secretory Club cells with high content of metabolizing enzymes in the terminal bronchioles of rodents [21]. Differences in the immune response to injury exist between human and murine eosinophil and neutrophil granulocytes, M1 and M2 macrophages [10]. Mucociliary clearance is faster in rodents, whereas supply with lymphatic vessels of the pleura higher in humans [21]. Cytochrome P450 enzymes are poorly active in humans in contrast to rodents and carboxylesterase is particularly inefficient in humans, not in rodents. Phase III enzymes, by contrast, are more efficient in humans than in rodents with the consequence that this particular clearance mechanism cannot be mimicked in animal studies.

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Physiological differences may play a role for the study of inhalation exposure. Rodents are obligatory nose and humans oro-nasal breathers, which leads to less particle filtering and higher delivered amounts to the human lungs. The burring of the nose in the fur may further decrease exposure in the rodents, but licking of aerosol deposited on the fur may increase exposure by oral uptake. Reflexes induced by the inhalation maneuver may mimic toxic action by decrease of ventilation rate and/or blood pressure upon stimulation of the parasympathetic system. Mice lack a cough reflex, which might induce toxicity and death by choking.

4.2. Animal models for the diseased lung

Identification of pathways and genes in the pathology of respiratory diseases as well as efficacy testing of drug require representative animal models. Similar to other cancers, screening of candidates for lung cancer treatment is performed on xenografts. The idea to personalize the model by using patient-derived xenografts has been discredited as alterations upon tumor progression in the animals do not resemble the patients' disease any more [22].

Animal models for respiratory diseases are in general induced rodent models. This means that the animals do not develop the disease naturally but need specific inducers to develop a phenotype with similar symptoms to the human disease. Natural models would be available by genetic variants in larger animals but are rare in rodents [19]. The best known model is the viable motheaten mouse for lung fibrosis characterized by elevated tumor necrosis factor-alpha (TNF-α) levels [23].

4.2.1. Animal models for cystic fibrosis

Cystic fibrosis pig and ferret are transgenics that mimic quite well the manifestations of human CF. The models were generated by disruption of the cystic fibrosis transporter (CFTR) gene or introduction of the ΔF508 CFTR mutation. Both species develop the typical phenotype in lung, pancreas, gallbladder and intestinal tissue. The transgenic mice are less suitable to study human CF because the animals lack spontaneous lung infections and manifestation in the pancreas. The presence of submucosal glands only in the proximal trachea of mice and absence in cartilaginous bronchial airways is regarded as major reason for the different presentation in the lung [24].

4.2.2. Animal models for asthma

The ovalbumin sensitization of mice for asthma is a typical example for an induced disease model. It comprises a sensitization step in the presence of aluminum hydroxide as adjuvant, and a second step where mice are challenged with the allergen introduced directly into the airways to induce asthma features [25]. The main difference between mouse models and human asthma is the fact that airway inflammation and airway hyperreactivity seem to resolve within a few weeks in the mice, while they continue in humans. House dust mites should in the future replace the ovalbumin because this allergen is more relevant for human asthma. Hyperreactive airways are seen in naturally occurring non-allergic asthma of horses and exercise-induced asthma of dogs. Cats, dogs, and sheep also develop allergic asthma [10].

4.2.3. Animal models for chronic obstructive pulmonary disease

In contrast to rats that do not develop COPD upon exposure to cigarette smoke, guinea pigs show the pathological changes of human COPD [19]. The model has the disadvantage that guinea pigs are more expensive than rats and that antibodies needed to study the physiological changes, are less available for this species. Murine models show considerable variation in expression of the COPD phenotype and considerable inter-strain differences. Although mice may react differently to humans because there is no mucus production in the murine bronchial tract [26], mouse model shows several similarities to

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human COPD, among others the more pronounced manifestation of the disease in females. Spontaneous COPD is seen in dogs and horses.

4.2.4. Animal models for lung infection & acute respiratory stress syndrome (ARDS, acute lung injury)

Pneumonia is very difficult to study in animal models due to species-specific pathogenicity of the microbes and differences in lung microbiota caused by specific pathogen-free (SPF) condition of the animals. SPF-housed animals do not have contact to disease-causing pathogens that can affect mouse health and research outcomes or to opportunistic and commensal organisms that typically do not cause illness in normal, healthy mice. The lack of contact to the variety of microbes present in the normal environment has marked influence on the immune system of these animals. Compared to wild animals they have a less activated immune system (antibody levels and circulating myeloid cells), which makes them more vulnerable to pathogens. The phenotype of T cells subsets in SFP mice resembles human neonatal blood, while T cells of mice from the pet store were similar to adult T cell populations [27]. Larger animal models are used for studying of infections with Mycobacterium tuberculosis (calves), *Chlamydia psittaci* (calves), *Pseudomonas aeruginosa* (sheep) and *Staphylococcus aureus* (sheep, pig). Spontaneous lung inflammations are observed in dogs, cats and horses.

Instillation of lipopolysaccharide (LPS) into rodents is a rather common model to cause ARDS. The model does not reflect the human pathology so far because animals either die within 72h after the LPS administration or they recover completely. This is in marked contrast to the human condition where changes are progressive and mortality is 20% in the first week and 40% after 4 weeks. In contrast to the small animals, pigs and sheep can spontaneously develop ARDS [10].

4.2.5. Animal models for lung fibrosis

The phenotype in rodents is most mostly induced by instillation of bleomycin, although other agents, hypoxic chloride, asbestos, silica, vanadium pentoxide, fluorescein isothiocyanate, lung irradiation, and species-specific viruses can also be used. Strain-specificity in the reaction to these toxicants, variability in fibrosis and high mortality represent the major limitation of the model [19]. Naturally occurring progressive lung fibrosis is seen in dogs, horses, cats and donkeys.

4.3. Aerosol exposure to animals

Not only should the anatomy, physiology and disease phenotype resemble the human situation, also similar amounts of drug or toxicants should be distributed in the relevant regions of the bronchial tree. In general, animals should receive the aerosol not by local instillation but by inhalation because the biological effect of inhaled particles is greater than that of instilled ones. This may be due to the more uniform distribution in the lungs upon inhalation and to the higher delivery to peripheral regions of the bronchial tree. The thin alveoles in the lung periphery have a greater capacity for absorption than the thicker and mucus-covered bronchial epithelium of the upper airways. Bypassing of the filtering by the nose by the intratracheal administration, on the other hand, results in high concentrations of the materials at the administration site, which may cause an inflammatory response and delay of lung clearance. The recommendation to use instillation only for dose finding and to back it up with nose-only inhalation is published in the OECD guidelines [28]. Out of the two most commonly used inhalation methods, the nose-only exposure is considered a better exposure method for rodents than whole body exposure because exposure occurs only be the pulmonary route, whereas in whole body exposure, oral exposure may occur because animals may lick aerosol that deposited on their pelts [29]. The airflow to each port of the nose-only exposure unit must exceed the

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minute ventilation rate of the animal in order to remove the exhaled air. Otherwise, if the animal re-breath exhaled air, oxygen concentration and exposure dose will decrease and carbon dioxide concentration increase. Change in ventilation rate of the animal either by stress or by sedation will influence the inhaled dose. Whole body exposure induces less stress because the animals are not restrained and is suitable for chronic inhalation studies. The disadvantage of this exposure is that the dose is difficult to determine. Aerosol may stick to the exposure chambers, the animals may prevent exposure by covering their noses and aerosol will deposit on their pelts.

4.4. Dose selection for animal experiments

Calculation of the drug dose to be used in clinical trials and determination of the therapeutic window (the difference between effective and toxic dose) require allometric scaling. Allometric scaling is an accepted method because body mass to surface area, lung mass to body mass and lung surface to body mass correlate in a linear way for mouse, rat, guinea pig, monkey, dog and humans [30]. Deposition fractions not taking particle size into account are assumed as 10% for rodents, 25% for canine, 30% for non-human primates and 40% for humans [31]. Since larger animals usually have slower metabolisation than smaller mammals, systemically acting drugs are scaled with a fixed exponent of 0.67 from rodents to humans. A study compared the deposited and effective deposited dose of orally inhaled salbutamol, budesonide, ipratropium and mometasone in mice and rats to humans and obtained exponents of 0.44, 0.60, 0.95 and 0.78, respectively [32]. The average of 0.69 showed that also for inhaled drugs the effective doses in larger are lower than in smaller species. For the calculation of toxicity, the effective deposited dose in animals is used as starting point. Allometric scaling is used to determine the efficient dose in humans, multiplication by 2.5 to account for the 40% deposition rate in humans (=projected effective human delivered dose). The safety margin is usually a factor of 10 and, therefore this dose needs to be multiplicated by 10. For the safety testing in animals, allometric scaling has to be applied again and finally the deposition fraction has to be taken into account by multiplication with 10 for rodents and 4 for dogs (rodents have 10% deposition fraction and dogs 25%).

4.5. Analyses of animal experiments

In addition to efficacy, animal experiments provide data on absorption, distribution, metabolisation excretion and toxicity (ADMET). In addition to effects on organs (histopathology, histological stains, immunohistochemical staining, in-situ hybridization, Western blot, PCR), changes in blood (whole blood count, serum chemistry, cytokine levels), biodistribution (tissue, plasma, urine analysis, scintigraphy, magnetic resonance imaging, computer tomography, ultrasound, positron emission tomography, single photon emission computed tomography) including excretion and changes body weight and in behaviour can be determined.

5. *In vitro* techniques in pulmonary research

In silico modeling by combination of lung deposition and physiologically based pharmacokinetic (PBPK) models is a good complementation of the cellular studies. Experimental data can be used as input parameters for the calculation of deposition, absorption, metabolism and excretion.

5.1. In vitro models for the healthy lung

Cellular models for respiratory cells have improved over the years compared to the previously used conventional culture (Fig. 2).

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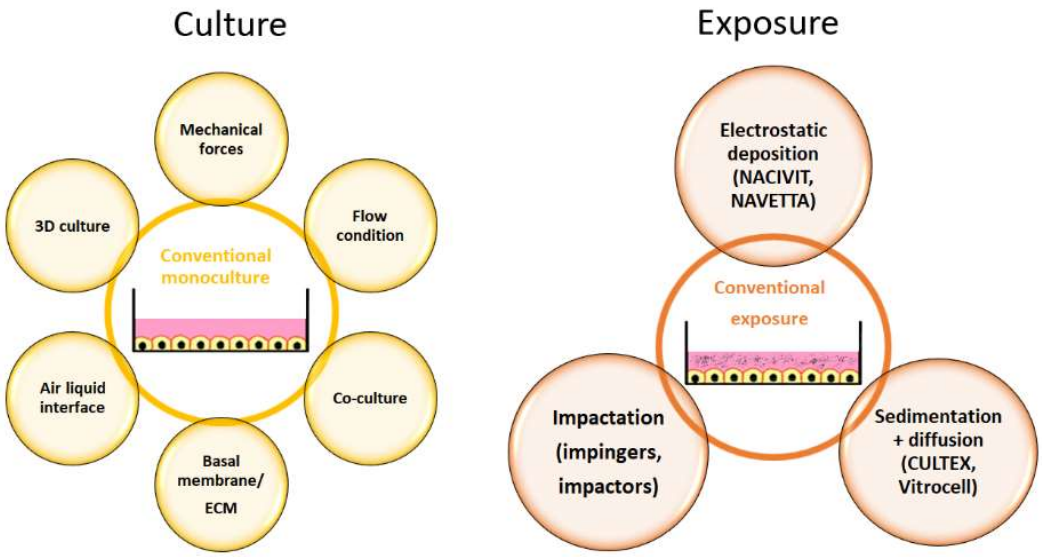

Figure 2. Adaptations of in cell culture (yellow) and exposure (brown) that result in physiologically more relevant *in vitro* models.

The supply with nutrients from the apical side is not physiological for most cells of the human body. Rare examples are endocardial endothelial cells, which receive O2 and nutrients by diffusion from the circulating blood at their apical surface. The important step in the development of models for respiratory cells was the invention of air-liquid interface (ALI) culture for a more physiologically relevant culture. ALI culture is characterized by cell growth on membranes and supply with medium only at the basal side. Respiratory cells are one of the few cells that physiologically are exposed to high O2 concentrations (104-108 mm Hg), whereas cells of inner organs experience concentrations of 28.9-88 mm Hg [33]. The polar environment increases cell differentiation and induces cilia formation in bronchial epithelial cells, expression of lung-specific metabolizing enzymes CYP2A13, CYP2F1 and CYP4B1, correct localization of the tight junction complex and function of sodium channel and CFTR [34]. The inclusion of more than one cell type (co-culture) is another important feature of the advanced lung models, although not all the 40 cell types presented in the lung can be included in the culture [35]. Researchers mostly co-culture in addition to epithelial cells, endothelial cells and fibroblasts also cells of the immune system, such as macrophages and dendritic cells [36]. If the entire tissue complexity is relevant, the use of precision cut lung slices as *ex vivo* model represents the best option. These samples, however, are stable only for a short period of time.

The choice of the model should be adapted to the specific research question. For cancer-related questions, the use of 3D culture to mimic the different zones of the tumor is essential, for the assessment of drug absorption or studies of local effects at the pulmonary barrier the presence of immune cells, perfusion and mechanical forces may be most relevant.

Due to the specific requirements, it is not surprising that researchers established or adapted existing models and/or read-out parameters in various ways. Use of primary cells, extracellular matrix, induced pluripotent stem cells are options to increase physiological relevance. Airway mechanics can be included by cell stretch devices, cell heterogeneity by precision cut lung slices, hemodynamics using perfused lungs, shear stress models and isolated vessel segments, barrier function by permeation assays, lung on a

chip models and perfused lungs. For the study of tissue remodeling specific read-out parameters, e.g. proliferation, differentiation, transdifferentiation, matrix deposition and juxta/para/endocrine signaling and for immunity and inflammation adhesion, migration, juxta/para/endocrine signaling and phagocytosis should be determined [10].

There are numerous home-made models that combine microfluidics, primary cells and 3D culture for instance for lung cancer [37,38] but this review will focus only the commercial systems because they are more suitable for standardized testing. Ready-to-use reconstructed tissues are available from MatTek Corporation (EpiAirway™ and EpiAirway-FT™) and Epithelix Sarl (MucilAir™, SmallAir™). The generation of home-made system can be standardized by using PneumaCult™ Expansion and Differentiation medium kits from STEMCELL Technologies [34]. There is also much activity in the design of commercially available lung-on-a-chip products [39]. The well-known companies Emulate Inc. and Alveolix AG but also other companies, such as NORTIS, Quorum Technologies (Artery-on-a-chip Vessel), MIMETAS, SYnVIVO, 4DESIGN BIOSCIENCES, and AIM BIOTECH develop chip solutions for human tissues. The ideal alveolar model would be a chip based on a mechanically active membrane and consisting of human cells cultured in ALI on extracellular matrix (ECM) on one side and endothelial cells on the other. The structural and mechanical functions of the ECM are pivotal for normal cell function and differentiation [40]. The ECM regulates passage of molecules, acts as local reservoir of growth factors and bioactive molecules and plays a key role in the development of respiratory diseases. The commonly used polydimethylsiloxane (PDMS) membranes, however, cannot act as reservoir but adsorb specific small molecules, which prevents assessment of permeation. Furthermore, fabrication of ultrathin and porous membranes to allow mechanical action and passage of compounds, is challenging [41]. All these issues probably contribute to the fact that no ready-to-use product is currently commercially available. Recently, Zamprogno et al. developed a material that may be suitable for commercialization of such devices [42].

5.2. Cellular models for lung diseases

Commonly used lung cancer models consist of lung carcinoma cells co-cultured with fibroblasts and immune cells. They are prepared by cell aggregation with or without scaffold in static or dynamic condition [43]. Different culture conditions (hypoxia, microfluidics, ALI, scaffolds) can be used. If the organoids are embedded in extracellular matrix, they form a lumen and drug candidates can be microinjected into the construct [44]. Organoids either generated from induced pluripotent stem cells or from primary cells isolated from patients are an important tool for drug screening. OncoCilAir™ tissues from OncoTheis, which are commercially available constructs on membranes, consist of bronchial epithelial cells, fibroblasts and non-small cell lung cancer cells (NSCLC), provide a greater level of standardization.

There are no standardized protocols, ready-to-use models or accepted alternative to *in vivo* tests for obstructive lung diseases, inflammation and pulmonary fibrosis. Examples for published setups to assess disease relevant parameters are listed below. Mucus flow in CF patient samples as indication for mucociliary clearance can be determined using transport of fluorescent spheres [45] or in reconstructed tissues (e.g. MucilAir™). CFTR activity can be determined in CF patient-derived nasospheroids in addition to mucus secretion and fluid secretion [46]. Alterations in asthma can be mimicked by wounding of primary bronchial epithelial cells from asthma patients compared to healthy controls [47]. Cytokines, particularly transforming growth factor-beta TGF-β1 levels are measured. A chip mimicking bronchial constriction has been developed for better mimicking the *in vivo* situation. COPD can by studied by repopularization of cadaveric scaffolds with normal or COPD patient-derived cells [48]. The process of EMT can be followed by exposure of Matrigel-embedded healthy bronchial tissue in ALI to cigarette smoke [49].

 doi:10.20944/preprints202105.0450.v1

The use of *in vitro* models could represent advantages over animal testing for infections because the species-specificity could be excluded [26]. For these studies co-cultures of bacteria and lung models and co-culture of *ex vivo* lung tissue with *Pseudomonas aeruginosa* biofilms to assess the impact of infection and the efficacy of antimicrobial agents could be used [50,51].

Pulmonary fibrosis was studied in a co-cultures of A549 alveolar epithelial cells, MRC-5 fibroblasts and THP-1 macrophages treated with bleomycin in a microfluidic system [52] and in pulmospheres generated from pulmonary fibrosis patients [53]. The pulmospheres can, for instance, be used as screening system for anti-fibrotic drugs. Fibroblasts cultured on extracellular matrix damaged by mechanically or oxidative stress serves as a model for lung fibrosis and aging [54]. Gel contraction induced by TGF-β stimulated A549 cells mimics the process of EMT [55].

5.3. Cell exposure to aerosols

Exposure of cells to aerosols is not easy because cell damage by the airflow has to be prevented. In contrast to the great variety of home-made models, commercially available exposure systems allow better standardization [56]. Available instruments imitate the mechanisms that also occur in the body, which are primary deposition based on impaction, sedimentation, and Brownian diffusion and secondary deposition in the turbulent flow of the upper airways by interception and electrostatic precipitation [14]. Impingers and impactors, used in particle characterization, separate particles based on impaction but cannot be used for cell exposures. This deposition is typical for the large (conducting) airways. Devices based on electrostatic impaction include the NanoAerosol Chamber for in Vitro Toxicity (NACIVIT), the Electrostatic Particulate Dosage and Exposure System (EPDExS) and the Novel ALI Exposure System (NAVETTA). These devices allow faster and higher (up to 100%) deposition efficacy compared to the 2% in CULTEX systems [57,58]. They are, however, not suitable for all particles because charge is needed. Sedimentation and diffusion as seen in the alveolar region of the lung are used in CULTEX CG and RFS, VITROCELL, Pharmaceutical Aerosol Deposition Device on Cell Cultures (PADDOCC) and Precise Inhale Xpose® ALI. A detailed description and illustration of the available exposure systems is provided by Karra et al. [14]. The most commonly used systems are commercialized by Cultex® Technology GmbH and by VITROCELL Systems GmbH. The Vitrocell® aerosol Exposure System consists of the vibrating mesh nebulizer Aeroneb® Pro, exposure chamber, cultivation module, quartz microbalance and heating [34]. The Vitrocell® Spiking System applies vapor mixed with air. Vitrocell® VC1 and VC10 and Borgwaldt® RM20S are smoking robots. CULTEX® RFS, CULTEX® RFS compact and CULTEX® LongTermCultivation-continuous use radial flow instead of the vertical flow used in the Vitrocell® systems.

5.4. Readout parameters of in vitro studies

Protein expression, cytokine release, gene expression, viability, metabolism, proliferation, migration, apoptosis, mitochondrial membrane potential, DNA damage, intracellular calcium changes, generation of reactive oxygen species are routinely used read-out parameters for *in vitro*/*ex vivo* experiments and inform about changes in cell physiology and intercellular interaction. Additional analyses may be required to study effects linked to a specific respiratory disease. TGF-β pre-treated cells can be evaluated for cell contraction, epithelial-mesenchymal transition, airway remodeling and elastin levels [55]. In membrane cultured cells and reconstructed tissues measurements of transepithelial electrical resistance and permeation can be analysed [59]. In spheroids, vascularization and cellular crosstalk are particularly relevant [60]. Reconstructed tissues/*ex vivo* samples are used to assess cilia beating frequency, mucus production, airway surface liquid volume, mucociliary clearance [61]. Effects on alveolar macrophages can be assessed by measuring of chemotaxis, phagocytosis and phospholipidosis [62].

 doi:10.20944/preprints202105.0450.v1

6. Lung deposition models and PBPK models

In silico models can support studies with analysis of deposition and ADME. Deposition models do not consider absolute deposited dose but the fraction of particles of a given size, shape and density that is deposited at a given region of the respiratory tract. Hofmann [63] classified the models in five groups and regarded lung morphology as the most important factor in the calculations. This classification differentiates between 1) semi-empiric models, 2) continuous or trumpet models and 3-5) truly mechanistic models. (Semi)-empiric models combine first principle mechanistic models with experimental data [64]. In the next sections only programs that are publically available, will be mentioned.

The International Commission of Radioactive Protection (ICRP) published a series of models, out of which ICRP66 is probably the best known [65]. It is an empirical regional compartment model and corresponds to a series of filters. The model indicates deposition resulting from inhalation and exhalation. Particle parameters size, density, hygroscopicity can be adjusted. For the biological parameters gender, ethnicity, physical activity, nose/mouth breathing, body weight can be adjusted. The most recent ICRP130 model includes also clearance from the alveoles to the blood. The National Council on Radiation Protection and Measurement (NCRP) published a very similar to the ICRP66 model, where another, presumably more correct, description of the deposition of nanoparticles is implemented. NCRP may better predict nanoparticle deposition pattern but clearance mechanisms may be better reflected by the IRCP130 model. Another similar to the IRCP66 model is the RADEP (Radon Dose Evaluation Program). The freely available Lung Dose Evaluation Program (LUDEP) is based on the IRCP130 regional compartment model. It allows modification of particle properties, tidal volume, FRC, breathing pattern, symmetric or asymmetric lung structure but not airway diameter and alveolar volume. Mucus transport of 20 mm/min in the trachea to 2 mm/min in the small airways is assumed. Translocation from the alveoli in other tissues is estimated as 0.1% of the deposited dose, and calculation of bone marrow, bone, lung, liver and gonad dose are possible [66].

Deterministic simple path models use symmetric branching and suffer from the lack of geometrical data in alveolar ducts and sacks. Typical paths for all five lobes have been implemented, which improved the performance of these models. The deterministic multiple paths models use typical paths and asymmetric branching pattern. Deposition is calculated for each single airway and some alveoles receive deposited particles from different paths. This may explain the preferential localization of lung cancer in specific parts of the lungs. In deterministic models simplified assumptions about airway geometry and airflow conditions are used to derive analytical solutions of air and particle motion. The model tracks the path of a population of particles within a bronchial tree. These programs are freely available and have used-friendly software. The most commonly used Multiple Path Particle Dosimetry (MPPD) and the Hygroscopic Particle Deposition Model provided by Helmholtzzentrum München (German Research Center for Environmental Health) are deterministic models. Morphologies of mouse, rat, rhesus monkey, sheep, pig and human lungs are available.

Mechanistic models determine particle transport and deposition by computational fluid and particle dynamics (CFPD). From an elementary viewpoint, CFPD can be seen as an extension of the well-known and established Computational Fluid Dynamics (CFD) knowledge, with additional modelling requirements to reflect the particle dynamics within the fluid flow. Stochastic multiple path models allow randomization of tube lengths, angles and diameters and perform runs for single particles. These routes differ for individual particles between the simulations but after simulation of hundreds of particles, an average pattern is obtained. In stochastic models, the morphology of the lung is

 doi:10.20944/preprints202105.0450.v1

considered to vary within certain limits in a random manner. Deposition fractions are derived from classical flow equations in the respiratory tract model, followed by particle behavior in that flow. The anatomical regions are seen as compartments with connecting flow, concentration and time properties and the airways are tubes that branch into finer airways and the way of splitting affects the model characteristics. There are basically two ways of particle tracking, Lagrangian and Eulerian. In the former single particles are tracked, which is often compared to a person traveling on a racket. In the Eulerian model an ensemble or a concentration of particles is tracked and this is described as a person standing on the ground and watching a group of rackets flying by. In these models, outputs are solved mathematically and are not based on assumptions from empirical model and fitting parameters. They are able to predict deposition at a localized level [67]. Some CFPD models include bronchoconstriction (airway diameter), emphysema (alveolar volume), elastic recoil, breathing conditions, lung clearance and mucus clearance. Despite the advances in this field due to increasing computing processes and imaging capabilities, most CFPD-based lung models address deposition in the upper airway regions only because of the limited availability of high quality *in vivo* data of the lower respiratory tract. Further, CFD simulations are computer intensive and need skilled users [68]. There are whole lung and site-specific models [66].

Trends in deposition as function of particle diameter and breathing conditions were similar in the comparison of five stochastic models but variation arose depending on the choice of central bifurcation geometry (branching angle, bifurcation shape), flow profiles and methods used in the derivation of the equations. Deposition fraction by diffusion and impaction were much more affected than sedimentation [69]. Multiple-path models are more realistic than semi-empirical models and deterministic single-path models and trumpet models because they are based on actual airway measurements rather than on average values. The current lack of a complete deterministic asymmetric description of the lower airways present the main limitation. In a more recent publication one dimensional cross-section (trumpet), deterministic symmetric generation (single-path), deterministic asymmetric generation (multi-path), stochastic asymmetric generation (multi-path) models and single-path CFPD were compared [70]. The same trends for particle diameters and breathing regarding regional bronchial and alveolar deposition, general lung deposition, lobar deposition, generational lobar deposition and generational surface deposition were identified. The author concluded that current deposition models correctly predict regional and generational deposition. The deposition fraction calculated by semi-empirical (IRCP66), trumpet, single-path, multiple-path and stochastic model varied only be 10% and showed a typical U-shape curve for all models. Regional differences in the deposition in the alveoli showed variation of 15% [71]. All models suffer from the lack of complete lung structure measurements and alveolar structures are extrapolated. The prominent inter-individual variability of 30-50% confirmed in experimental studies is realized in the models by scaling the linear airway dimensions according to body weight and height. Breathing pattern and ventilator rate are anticipated to be a major source of error. From the particle side, not only size but also hygroscopicity has also a prominent effect. Hygroscopicity is accounted for in the MPPD model [64].

The majority of models (IRCP, LUDEP, RADEP) were designed for environmental exposure. They underestimate oropharyngeal deposition of pharmaceutical aerosols due to the lower filtering in mouth breathing compared to nose breathing. The underestimation of oropharyngeal deposition by environmental deposition software programs can be improved by the use of mouth-throat replicas in combination with CFPD simulations. In general, MPPD appears to be good for toxicity studies and NCRP better to determine nanoparticle deposition.

 doi:10.20944/preprints202105.0450.v1

Data for ADMET are usually obtained from animal experiments. The process of absorption can be mimicked by permeation across cell monolayers because Papp values obtained in Calu-3 monolayers correspond well to permeability of the lung [72]. Other physiological parameters are available in data banks for the known compounds or molecules. With further improvements in the organ-on-a-chip technology it appears possible that also metabolisation and excretion data can be obtained from these systems instead of relying on data generated in animals.

Mimetikos™ Preludium (Emmace Consulting) contains calculation for regional distribution, dissolution, barrier permeation and mucociliary clearance. The programs allow to adjust default settings based on data, obtained from other deposition programs or from experimental data (e.g. precision cut ling slices) [73]. The only commercially available program that combines mechanistic models for deposition, absorptive and non-absorptive clearance in an anatomical representation of the lung and PBPK modeling is the GastroPlus™ Nasal-Pulmonary Compartmental Absorption and Transit Models from SimulationsPlus Inc. [74]. The model consists of three lung compartments and one extrathoracic compartment, each of them with epithelial/tissue and airway liquid compartment. Deposition is calculated by ICRP66 model and dissolution by Noyes-Whitney principle. The particles deposited in the extrathoracic compartment are cleared to the gastrointestinal tract and oral absorption assumed. Mucociliary clearance, lung metabolism and mucus binding are integrated in the software. PBPK description is missing for the other models, Simcyp™ Simulator and PK-Sim. PulmoSim™, developed from SimCyp® by Pfizer, divides the dose into two fractions, one to the lung, the other to the gastrointestinal tract. Mucociliary clearance, dissolution, absorption, tissue binding, systemic distribution and clearances are included but no data are available for pulmonary and systemic exposures. SimCyp® Simulator (Certera) is based on a first order non-mechanistic inhalation model, where deposition is not calculated. The alveolar dose is treated like intravenous dose and the dose to the conducting airways like delayed oral dose. PK-Sim (Bayer AG) is used to generate the systemic profiles.

7. Conclusions

Screening for inhaled compounds, safety testing and assessment of environmental toxicants suffers from the lack of appropriate animal models and limitations in animal inhalation exposure. There are, on the other hand, good *in silico* predictions for aerosol characterization and deposition and cellular/tissue models that could be used to predict effects in humans. Major limitations are that differences in anatomy, physiology of upper and lower airways need models representing both regions for complete assessment of pulmonary effects. Further, the assessment of repeated exposure in a physiologically relevant model is difficult. Co-culture on chips appears problematic because epithelial cells prefer laminin-rich matrices, whereas endothelial cells and mesenchymal cells prefer collagen-rich surfaces [26]. The inclusion of mechanic forces is possible but only relevant when the respiratory part of the airways (e.g. alveoles) is studied. Basic ALI airway models, consisting of respiratory cells only, are stable for prolonged times. To make these models more relevant, immune cells should be included. This, however, may affect the stability of the model because the cell types have different requirement for survival and growth. A major hindrance for the broad acceptance of these systems is the fact that they have to be constructed using rodent cells in order to be able to validate them with the animal models [34]. Despite the availability of a great variety of these models, experts do currently not advocate the only use of these studies for pulmonary research [10].

Funding: This research received no external funding.

Acknowledgments: In this section, you can acknowledge any support given which is not covered by the author contribution or funding sections. This may include administrative and technical support, or donations in kind (e.g., materials used for experiments).

Conflicts of Interest: The author declares no conflict of interest.

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