Meeting Report

Second Virtual Summer School: Alternative Methods in Science: Towards Model Complexity

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The international Second Virtual Summer School: Alternative Methods in Science: Towards Model Complexity (https://amsc.lakecomoschool.org/), addressing young researchers from different disciplines, was focused on the application of new, complex methodologies and models to implement and encourage innovative applications and perspectives in science. The two-day on-line summer school, held on June 7-8, 2021, was chaired by Francesca Caloni, Università degli Studi di Milano, Department of Environmental Science and Policy, and was attended by 31 participants from all over the world.

Francesca Caloni opened the summer school, explaining the possible interpretations of the term "complexity", which can be considered in relation to models, systems, the environment, mixtures of toxicants, and the multiple disciplines involved. Complexity was associated with examples on the application of alternative methods, considering complex models such as spheroids or organoids, but also complex *in vitro* approaches or strategies. How to promote awareness of the 3Rs, moving to a predictive, interdisciplinary science, with new and controversial aspects, was discussed.

Helena Kandarova, CEM & FChFT Bratislava, gave a lecture entitled "How much complexity is enough? *In vitro* phototoxicity case". Phototoxicity is defined as a toxic response that is elicited after the first exposure of skin (or other organs) to certain chemicals and subsequent exposure to light, or that is induced, similarly, by skin (or other organ) irradiation after systemic administration of a chemical. *In vitro* phototoxicity testing (the 3T3 NRU PT), performed with a mouse fibroblast cell line, was one of the first tests implemented in a European regulation (EU B.41), and later also in OECD TG 432. Information derived from the *in vitro* 3T3 NRU phototoxicity test serves to identify the phototoxic potential of test substances, i.e., the existence or absence of possible hazards likely to arise from a test substance in association with exposure to UV and visible light. Despite extensive validation, when used across the various industrial sectors, scientists found that the method has several limitations. Testing of poorly water-soluble materials or formulations is difficult and, especially for pharmaceuticals, the 3T3 NRU PT provided too many false positive results not confirmed in *in vivo* studies. Another drawback of this method is a lack of predictivity of phototoxic potency. To overcome these problems, scientists already in 1994 suggested the use of reconstructed tissue models, either in a stand-alone or as a second-tier test in the phototoxicity testing strategy. 3D skin models were, however, only implemented in the ICH S10 guideline on the phototoxicity testing of pharmaceuticals in 2015. A new OECD TG on phototoxicity testing was endorsed at the OECD WNT in April 2021 and will be published shortly. The presentation summarized the long way from development to acceptance of a testing concept utilizing 2D and 3D *in vitro* models and briefly discussed the benefits as well as drawbacks of models and testing strategies with various levels of complexity.

Laura Ceriotti, in collaboration with Marisa Meloni, Vitro-Screen, Milan, Italy, presented a lecture entitled "Skin irritation potential and biocompatibility of complex mixtures". Skin irritation refers to reversible damage to the skin occurring after exposure to a substance or a mixture. OECD TG 439 was introduced in 2010 as an *in vitro* test method for the hazard identification of irritant chemicals to replace the *in vivo* rabbit skin irritation test or to be used as a partial replacement test within a testing strategy. OECD TG 439 is applicable to solids, liquids, semi-solids, and waxes and is theoretically applicable to both substances and mixtures, even if limited information on mixture testing is available and results obtained with mixtures should be evaluated carefully (e.g., botanicals). Biocompatibility is a more general term identifying the tests required by ISO 10993 for evaluation of the safety of materials used in biomedical devices. One of the toxicological endpoints to be considered is irritation, in particular skin irritation (ISO 10993:1). Medical devices, however, include a broad range of materials. Solid, insoluble polymeric materials cannot be tested as such but need to be extracted in solvents (polar and apolar) prior to testing (ISO 10993:12). In such complex extracts, concentrations of chemicals, contaminants and/or leachables may vary or be very low, and thus may be difficult to detect and identify. To develop an alternative protocol to the *in vivo* skin irritation assay that would be sufficiently sensitive to assess the irritation potential of medical device extracts, the incubation time used on RhE methods defined in OECD TG 439 was extended to 18-24 h. The adapted protocols were shown to be suitable for the detection of irritants in diluted polymeric mixtures (De Jong et al., 2018) and can be used as first instance to assess the irritation potential of medical devices and their extracts (new ISO 10993:23). The presentation included, as a case study, the evaluation of the irritation potential of secondary raw materi-

als, which are an interesting example of complex mixtures owing to their chemical-physical characteristics and potential presence of contaminants or leachables derived from their origin and the recycling process.

Arno C. Gutleb, Environmental Research and Innovation (ERIN) Department, Luxembourg Institute of Science and Technology, gave a lecture entitled: "How complex should an *in vitro* model be?". Classically, *in vitro* models were based on single cell-type based models, cultured submerged in a plastic well. In recent years, complex models using more than one cell type have become more and more common. Such complex models need careful characterization of the properties of the cell types used for their assembly. However, there is more than just the individual cell type property that needs to be characterized, as cells start to change properties and their behavior in the presence of other cells, e.g., gene expression and functional responses start to change under such conditions. In addition, the similarities and discrepancies between 3D-models and human *in vivo* tissue need to be understood. Models should be as complex as possible and as complex as necessary to mimic physiological responses (Marescotti et al., 2019).

Hassan Rashidi, UCL Great Ormond Street Institute of Child Health, University College London, showed the "Pros and cons of adding an extra dimension to predict drug-induced hepatotoxicity". There is a high attrition rate of promising drug candidates in post-marketing stages due to adverse drug reactions (ADRs). While animal safety tests were introduced as a mandatory requirement before a compound can proceed into clinical development, such animal models are often poor predictors for human toxicity events due to interspecies differences, e.g., in liver-specific functions. The use of primary human hepatocytes (PHHs) is considered the gold standard in *in vitro* models for drug toxicity testing. However, their scarcity and transient *ex vivo* phenotype has limited their wide-spread application in predicting toxicity in pre-clinical drug development. Consequently, a variety of alternative 2-dimensional (2D)-models have been developed, including platforms based on hepatoma cell lines and human pluripotent stem cell (hPSC)-derived hepatocyte-like cells. Although these models have allowed for the *in vitro* analysis of several mechanisms of DILI, such as oxidative and endoplasmic reticulum stress and mitochondrial toxicity, none has been shown to accurately predict drug-induced liver injury (DILI). Recently, a novel platform to generate hPSC-derived 3D hepatospheres under xeno-free and GMP-ready conditions was developed (Rashidi et al., 2018). Unlike their 2D counterpart, the 3D hepatospheres downregulate expression of α-fetoprotein, a fetal marker, and remain metabolically active and drug-inducible for over a year in culture, providing a better *in vitro* platform to evaluate long-term effects of new lead compounds in a more physiologically relevant setup. In addition, hPSC-derived 3D hepatospheres showed higher sensitivity to drug toxicity compared to 2D counterparts.

Yula Sambuy, gave a presentation entitled "Advanced models of the intestinal mucosal barrier". The epithelium of the intestinal mucosa represents one of the most extensive barriers between the external environment (the intestinal lumen) and the internal organs. It is a major site of exposure to potentially toxic substances deriving from the diet, food additives or oral therapeutic agents. The intestinal mucosa is particularly important in toxicology, both as a direct target and as an indirect route of entry of xenobiotics or allergenic substances that may be harmful at the systemic level. Advances in cell culture techniques have led to the establishment of *in vitro* models of the intestinal epithelium, reproducing many of the structural and functional features of the *in vivo* epithelium. Among these, the most widely used is the human intestinal Caco-2 cell line, capable of differentiating on permeable substrates into a monolayer of polarized cells, coupled by functional tight and adherence junctions and expressing several transport and metabolic features of the absorptive enterocytes of the small intestine. However, this and other similar models are derived from tumor cells and lack some of the control pathways and the tridimensional organization of normal tissue. More recently, 3D-intestinal preparations from normal human tissue have become available for toxicity, metabolism, and drug absorption studies. A further step forward in the development of human tissue-relevant models of the intestinal mucosa, incorporating the different cell types that interact in the formation of a functional mucosal barrier *in vivo*, is emerging from the most recent advances in the use of induced pluripotent stem cells (iPSC). The added complexity and higher adherence to the *in vivo* situation of these models will contribute to the improvement of their predictivity in toxicological and translational research.

The lecture presented by **Teresa Coccini**, Laboratory of Clinical and Experimental Toxicology, ICSM Maugeri, IRCCS Pavia, Italy, was entitled "Human umbilical cord-derived neural-like cells: towards model complexity for neurotoxicity screening". Human cell-based models are strongly recommended as relevant alternative methods to reduce the uncertainty in species-specific extrapolation of results and to improve prediction in toxicology. In this respect, the use of stem cells (SCs) currently represents one of the emerging trends in technologies for developing assays and tools. SCs can be used to generate large populations of stably differentiated cells representative of different target species, including humans, and provide a non-transformed source of cells that can be differentiated into any lineage and serve as potent *in vitro* models. Successful differentiation of SCs into neuronal lineages is widely reported, and recent data confirm that the mesenchymal stem cells derived from human umbilical cord can transdifferentiate into neuronal-like cells (hNLCs). These properties make hNLCs a potential gold standard tool for establishing *in vitro* models of neurotoxicity. Examples applying these human NLCs (from 2D cell-based model and moving to 3D spheroids) associated with a complementary test panel after exposure to different compounds (e.g., nanoparticles, new psychoactive substances) were presented in the lecture. These findings support the value of using such models in the first step of hazard identification for xenobiotic risk assessment through a screening strategy to define neurotoxic effects of emerging toxic compounds in terms of mechanistic understanding of cellular responses.

Doris Wilflingseder, Institute of Hygiene and Medical Microbiology, Medical University of Innsbruck, presented "Applying advanced infectious disease models to study SARS-CoV-2". The growing spread of emerging infectious diseases such as COVID-19 or resistant pathogens indicates the need to speed up research on finding prevention or novel treatment options and testing novel innovative compounds. Since effective drugs or vaccines must induce both humoral and cellular responses against pathogenic challenges, novel alternative human approaches are needed, and improved methods for delivery must be tested. Rapid developments in high content screening as well as organotypic cultures provide groundbreaking new tools to study pathogen transfer at entry sites or to test repurposed drugs and novel vaccination strategies. Optimized intelligent human barrier models combined with infection-relevant immune cells and humoral components may be used to characterize and hinder overshooting host responses, pathogen entry, and initial transmission steps within a 3D system. These human systems offer improved power to test delivery methods, adjuvants, repurposing of drugs or novel vaccination approaches in high throughput.

Sonja von Aulock, Editor of ALTEX – Alternatives to Animal Experimentation, concluded the Summer School with a topic of great interest to researchers at early stages of their career: "Promoting the 3Rs in scientific writing and publishing". Many scientists working in the broad field of biomedicine can contribute towards protecting animals and promoting the 3Rs, even when their research is not primarily about replacing, reducing or refining animal experimentation. Such contributions can include planning a research project based on a thorough literature search that encompasses non-animal approaches and considers the 3Rs, choosing to use materials that do not involve animal suffering where possible, seeking to improve the welfare of animals used for research beyond minimum guidelines, documenting research according to best practice, reporting research according to guidelines that help to ensure the results can be evaluated and replicated by others, critically evaluating own research and pointing out its limitations, looking for potential applications of results in the 3Rs field, publishing relevant research in journals promoting the 3Rs, and considering and advocating these points when reviewing articles for journals, ethical boards and funding. The journal ALTEX has gained a high level of recognition in the field of alternative methods and beyond. It promotes the 3Rs by stringent selection of articles based on their potential 3R impact, by requiring and checking that any animal experimentation is reported according to the ARRIVE guidelines, by raising awareness on the use of materials whose production involves animal suffering, by implementing a critical and unbiased peer-review process, and by publishing all its content open access to make it available to all interested parties.

At the end of the presentations, the participants were invited to express their opinion on the complexity, *in vitro* models, and future of replacement.

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