Meeting Report

CellTox Days 2022 – Inside the Barriers: *In Vitro* Models and Their Applications

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The board of the Italian Association for *In vitro* Toxicology, CELLTOX, organized CellTox Days 2022 "*Inside the barriers: in vitro models and their application*", four webinars held during February and March 2022 dedicated to innovative *in vitro* solutions and technologies applied to biological barrier research.

Barriers play fundamental roles in maintaining homeostasis by regulating development, acting as the first line of defense in chemical and physical immune protection, and defining the frontier between the internal milieu and the outside environment (skin, gastrointestinal tract mucosa, lungs, etc.). They protect cell populations, tissues, or niches (blood-brain barrier, placenta, etc.), allowing liquid, solute, gas, and nutrient exchange between tissue compartments. Many diseases affect the physiology of these barriers and thus impact homeostasis.

Drug development is still characterized by a high rate of attrition, in part due to the poor translational value of animal-based models owing to interspecies discrepancies. Progress has been made in the development of human-relevant new approach methodologies of human barriers for disease modeling and drug development applications.

Day 1: Modelling the skin

Elena Dellambra, IDI-IRCCS, Rome, Italy, gave a lecture entitled "*In vitro skin models and their applications*". Cell-based therapy is a strategy aimed at replacing or repairing severely damaged tissue with cultured cells. Under appropriate culture conditions, human epithelial stem cells generate cohesive sheets of autologous stratified epithelium suitable for clinical purposes. For instance, transplantation of autologous epidermal sheets allowed life-saving epidermal regeneration for patients suffering from massive full-thickness burns (Pellegrini et al., 1998), sheets bearing melanocytes induced permanent repigmentation in patients suffering from vitiligo and piebaldism (Raskovic et al., 2006; Guerra et al., 2003; Bondanza et al., 2007), and gene correction of epidermal stem cells allowed the regeneration of a fully functional epidermis in patients suffering from junctional epidermolysis bullosa (Dellambra et al., 2001, 1998; Hirsch et al., 2017). The inability of 2D cell culture systems to adequately reproduce the native structure and function of skin led to the development of three-dimensional (3D) skin models composed of epidermal and dermal layers. These skin equivalents have been used as therapeutic tools for large and chronic skin lesions and as suitable alternatives to animal models for preclinical research and drug discovery purposes (Dellambra et al., 2019). Advances in tissue engineering, in particular iPSC technology, microfluidic platforms, and 3D bioprinting, allowed to produce next-generation skin equivalents. The integration of skin equivalents with other skin cell types (e.g., immune, neural and vascular cells, adipocytes) and new scaffolds/matrices provided new 3D models. Remaining challenges include the simulation of native skin structure (e.g., true blood vessels, nerves, and mature skin appendages) (Dellambra et al., 2019; Weng, et al., 2021) and modeling of skin functions such as wound repair, temperature control, and sensation.

Francesca Delle Monache, IDI-IRCCS, Rome, Italy, presented "*3D bioprinting of human skin and squamous cell carcinoma (SCC) as advanced models for precision medicine*". Scaffolds represent a fundamental aspect of engineering skin equivalent models to better mimic the skin barrier. SCC cell lines have been used for studying pathogenetic mechanisms and drug screening, but 2D SCC cultures lack microenvironment networks. Cancer-associated fibroblasts (CAFs) remodel the tumor microenvironment, playing an active role in SCC development. A 3D SCC model was developed by seeding SCC cell lines on a biodegradable polymer substrate, incorporating CAFs from human biopsies, and exposing the organotypic cultures at the air-liquid interface (ALI) in order to promote full epidermal differentiation and stratification (Panacchia et al., 2010; Maurelli et al., 2006). This 3D model has been used to investigate dysregulated pathways in SCC initiation and promotion and to assess potential pharmacological targets (Dellambra et al., 2021; Tinaburri et al., 2020). Since SCC 3D models do not fully reflect the architecture of skin cancer tissue, 3D bioprinted human skin and SCC models were generated as advanced models for precision medicine. Indeed, 3D bioprinting allows the *in vitro* production of engineered biological tissues by homogeneous layer-by-layer deposition of biological "inks" (cells, growth factors, and other biomaterials) that imitate natural tissue characteristics (Weng et al., 2021). Protocols for skin and SCC models by 3D bioprinting were optimized in collaboration with Università Campus Biomedico, Rome, by adapting the extrusion BIOX bioprinter to obtain a bioink with good printing adaptability, high biocompatibility, and good mechanical stability.

Tommaso Sbrana, IVTech Srl, Massarosa, Italy, presented the lecture "*New tools for advanced in vitro models*". Dynamic bioreactors play a crucial role in mimicking physiological fluid movements and dynamics as well as sustaining cell viability. In a collaboration with the University of Verona, a millifluidic bioreactor composed of the LiveBox 2 device customized to house skin explants with an ALI module and a lower fluidic compartment was developed (Cappellozza et al., 2021). After 48 h of culture of full-thickness rat skin in the device, annexes and mi-

crostructures were preserved contrary to culture in static conditions, which resulted in loss of tissue integrity, cell necrosis, damaged mitochondria, production of lactic acid, and presence of urocanic acid on the basal side of the skin, signs of a loss of tissue integrity. This study demonstrated the importance of the fluid dynamic conditions to sustain tissue integrity, viability, and function. This dynamic platform was also used with human skin-derived biopsies as a model for cosmetics testing or for studying skin pathologies.

Silvia Scaglione, React4Life, Genoa, Italy, gave a lecture entitled "*A novel fluidic microphysiological system for multiple 3D tissue culture towards more predictive drug testing*". A novel multi-organ microphysiological system (MIVO®) was used to culture different 3D human tissue models (e.g., skin, gut, liver, tumor tissues) and to test the pharmacokinetics and pharmacodynamics of different molecules (therapeutic agents). Tissues of biologically relevant size (up to 5 mm) or biopsies were cultured within the MIVO chamber under blood capillary fluid flows that allow to (i) feed the 3D tissue, (ii) resemble cell migration and infiltration (Cavo et al., 2018), and (iii) transport therapeutic molecules by recapitulating either oral drug administration or the systemic route (Marrella et al., 2020, 2021). Additionally, circulating immune cells can be co-cultured under physiological culture conditions to investigate their crosstalk with other healthy/pathological cells (Vitale et al., 2022). The diffusion of reference compounds was evaluated by culturing both skin biopsies and biomimetic membranes in the MIVO, according to OECD guidelines. This study highlighted highly reliable and predictive penetration kinetics of hydrophilic and lipophilic molecules; moreover, the reproducibility of results was much improved over other diffusive chambers, opening new perspectives for dermal absorption (Pulsoni et al., 2022).

Silvia Letasiova, MatTek IVSL, Bratislava, Slovakia, gave a presentation entitled "*EpiDerm Phototoxicity test – part of new OECD TG 498: Reconstructed Human EpiDermis Phototoxicity test method*". The 3T3 NRU PT (OECD TG 432) provides a high level of sensitivity, however, it generates a high rate of false positive results due to its lack of the barrier properties of natural human skin or other targeted tissues. An *in vitro* phototoxicity test using the human reconstructed epidermis model EpiDerm™ (EpiDerm™ H3D-PT) was developed and pre-validated almost 20 years ago and now can be used either as a stand-alone method for phototoxicity testing of topically applied materials or in combination with the 3T3 NRU PT to minimize false positive results from this assay. In June 2021, OECD TG 498: *in vitro* Phototoxicity: Reconstructed Human Epidermis Phototoxicity test method was adopted as a stand-alone method for evaluating the phototoxic potential of a test chemical after topical application in reconstructed human epidermis (RhE) in the presence and absence of simulated sunlight. EpiDerm™ H3D-PT is currently the only tissue model accepted under this test guideline.

Alice Cattaneo, Chemservice, Milan, Italy, delivered a lecture entitled "*Skin irritation and corrosion: in vitro studies with reconstructed tissue*". The previously mentioned EpiDerm™-based model (human epidermis), which consists of human epidermal keratinocytes forming a multilayered reconstituted tissue, was used to determine cell viability after exposition to various treatments using the MTT assay. Skin irritation or corrosion protocols were optimized to better classify the tested substances working with GLP principles.

Day 2: Modelling the pulmonary barrier

Alessandra Ludovico, Istituto di Biofisica-CNR, Genoa, Italy, gave the presentation "*Airway epithelial models for chemical compounds screening*". The airway epithelial model consists of a pseudo-stratified polarized epithelium. The model was used to better understand the effect of molecules used for the treatment of cystic fibrosis (CF). Although CF is a multi-organ disease, it mainly affects the respiratory system. CF is caused by mutations of *CFTR*. The CFTR protein is expressed on the apical membrane of airway epithelial cells, pancreas, and other tissues and is involved in chloride-bicarbonate exchange. Defective CTFR leads to an alteration in salt and water homeostasis and causes the production of very thick mucus in the airways, leading to recurrent infections and antibiotics resistance. Epithelial ALI culture allows to examine bronchial tissue, which produces mucus after a few weeks in specific culture conditions. Changes in the chemical-physical properties of the mucus in response to different treatments can be assessed. In addition to viscosity, studied with multiple particle tracking technique, osmolarity, pH, protein concentration, and variations in air surface liquid (ASL) height above the epithelium can be examined.

Thomas Hartung, Johns Hopkins Center for Alternatives to Animal Testing, Baltimore, MD, USA, presented "*Microphysiological systems (MPS)* – in vitro *on steroids*". New cell culture technologies have become more broadly available for overcoming the shortcomings of traditional culture. These include the use of stem cell-derived cells, cocultures of different cell types, scaffolds and extracellular matrices, perfusion, 3D cell culture, tissue architecture, and organ functionality. Prominent technologies include tissue slices, layered cultures, transwell cultures, hanging drop cultures, the use of scaffolds, 3D bioprinting, spontaneously aggregating cultures, and re-aggregating cultures. Increasingly, multi-organ-on-chip systems combining such cultures through microfluidics are being developed. The physiological relevance is further enhanced by the measurement of biomarkers (e.g., key events of pathways) and more holistic assessments of cell responses using high-content methods. These approaches are still rarely combined to create MPS, and in fact not all cell culture needs to be that sophisticated. Regulatory toxicology has only slowly begun to embrace these new approaches. However, major areas of toxicology have not yet found *in vitro* solutions. The lessons learned from the development, validation, and acceptance of alternative methods augment the ongoing creation of quality assurance of MPS. The recently drafted Good Cell and Tissue Culture Practice (GCCP) 2.0 (Pamies et al., 2022), which expands the original GCCP guidance from 2005 to include these new approaches, and Good *In Vitro* Reporting Standards (GIVReSt) (Hartung et al., 2019), under development, support their implementation. The advent of microphysiological sys-

tems represents a key scientific opportunity to develop more human-relevant test systems and serve society. They are forming a scientific field with dedicated conferences, societies, journals, best practices for culture (GCCP 2.0) and reporting standards, and educational offers.

Annamaria Colacci, University of Bologna, Arpae Emilia-Romagna, Italy, gave the lecture "*In vitro models for the study of the response to exposure to environmental pollutants*". Exposure to environmental toxicants is responsible for about 9 million deaths. COPD represent the major pathologies observed after either acute severe air pollution episodes (SAPE) or chronic exposure via the airways. Studies were performed to investigate air pollution profiles in the Po valley and to better define their sources and mechanisms of action. The A549 cell line was used because it mimicks a number of pulmonary functions and exhibits enzymes and transport proteins involved in the biotransformation/detoxification of xenobiotics. 12 m^3 air samples (including PM2.5 and PM1) were collected, and A549 were exposed to their organic and non-organic extracts. The transcriptome analysis showed significant dysregulation of pulmonary homeostasis (alteration of the epithelial barrier, activation of AhR, activation of genes involved in the inflammatory response, and TH_{17} cytokines) and oxidative stress (NO and iNOS).

Jan Markus, Mattek IVLSL, Bratislava, Slovakia, gave a presentation entitled "*Inhalation toxicity assays using the EpiAirway in vitro airway model*". The EpiAirway model resembles the tracheal-bronchial epithelial tissue. Cells composing this engineered tissue organize as a pseudo-epithelium and express tissue-specific structures, characteristics, and functions (cilia structures, mucus production, barrier function, etc.). This well-differentiated model can be used in pharmaco-toxicological and pathophysiological research. A study of the inhalation toxicity of e-cigarette aerosols and tobacco on cell homeostasis was performed by assessing various parameters including viability and TEER (Neilson et al., 2015) and found that e-cigarette aerosols seemed to be less toxic than cigarette smoke aerosols. This model could represent a potent tool to classify chemicals causing potential toxicity after acute inhalation, and evaluations are underway to better characterize the model. The reconstructed tissue has also been employed to model respiratory infections such as SARS-CoV-2 infection.

Day 3: Modelling the gastrointestinal barrier

Francesca Rescigno, Vitroscreen srl, Milan, Italy, gave a lecture entitled "*Intestinal barriers: from 3D epithelial models to intestinal spheroids*". Several assays have been established on 3D reconstructed epithelial models to evaluate barrier function integrity of mouth and oral mucosa, oesophagus, and intestine. 3D oral epithelium impairment can be investigated by TUNEL (detection of apoptotic cells). The oral barrier integrity was improved in the presence of commensal bacteria such as *S. aureus* colonies forming biofilm. Continuing along the gastrointestinal (GI) tract, the integrity of oesophageal epithelium could be investigated by studying the expression and localization of tight junction (TJ) proteins, key markers of barrier integrity. Film forming assays also represent a strategy to assess GI barrier impairment. Epithelial permeation can be evaluated by measuring the adsorption and penetration of small molecules such as caffeine. A novel intestinal micro-physiological model based on scaffold-free spheroids grown using hanging drop technology was also developed. It mimics all main features of the human intestine, with enterocyte turnover, an intense cellular metabolism, and TJ expression. The model can be made more complex by exposure to conditioned media from immunocompetent cells or intestinal bacteria secretomes, and it is suitable as a physiological screening platform for new therapies and human care solutions.

Jan Markus presented "*An in vitro model of human intestinal epithelium for studying drug toxicity and pharmacokinetics*". A 3D reconstructed tissue composed of human-derived small intestine epithelial and endothelial cells and fibroblasts grown at the ALI was developed. The model displays brush border, tight junctions, mucus production, barrier function, transport proteins, and drug metabolizing enzymes and can be employed to study the response to drugs or highly toxic chemicals. The EpiIntestinal model also can be used for studying drug permeation, drug metabolism, inflammation, and immune response as well as response to microbial infection.

Silvia Scaglione gave the lecture "*2D/3D intestinal biomimetic tissue models combined to microphysiological system for in vitro absorption assays*". The MIVO platform was combined with EpiIntestinal reconstituted tissues to reproduce the intestinal barrier and its dynamic flow. This combination presented a more relevant model to predict gut absorption and metabolism compared to *in vivo* organs (Marrella et al., 2020). The model has also been used to study tissue healing in response to drugs or probiotics in disease or injured tissues.

Francesca Piccapane, University of Bari, Bari, Italy, presented her research on "*A fluidic platform to implement advanced in-vitro models of pathophysiological intestinal barriers*". Epithelial cells grown as conventional 2D cultures on plastic or porous supports in static conditions do not faithfully reproduce native physiology. This can be improved by mechanical stimuli such as shear stress, achieved by a continuous flow of culture medium at the apical or basolateral side, known as dynamic conditions. IVTech devices were used to assess how dynamic culture conditions affect cellular integrity of the Caco2 cell line by evaluating morphology, integrity of tight junctions, and actin-based cytoskeleton by immunofluorescence analysis of cells grown on porous filters inside the LiveBox 2 and subjected to tangential flow both at the apical and basolateral side. The cells formed a much higher and larger columnar epithelium than in static conditions. To simulate the composition of the colonic epithelium *in vivo*, *in vitro* coculture models were created by combining Caco2 cells, as an enterocyte model, and HT29MTX-E12, as a mucus-secreting cell model, in a ratio of 90:10 or 70:30 (Caco2/HT29MTX-E12). Compared to monolayers grown under static conditions, TEER was increased in monoculture and coculture grown under dynamic conditions, suggesting that the application of mechanical stimuli improves the formation of functional, tight monolayers.

Day 4: Modelling the multiorgan

Silvia Scaglione presented "*A new immunocompetent OOC platform for modelling the 3D tumor microenvironment with capillary flow-driven immune cells*". The MIVO system was adopted to test the efficacy of an anticancer drug placed in the fluidic circuit, mimicking the systemic drug route. The regression of the ovarian tumor (SKOV-3 cells) was observed and measured within the MIVO device (Marrella et al., 2021). Results were in line with *in vivo* data and were achieved faster (time reduction 75%). Similarly, a human 3D neuroblastoma model (Marrella et al., 2019) was optimized to develop a tumor/immune cell coculture within the MIVO as an immune-oncology screening platform. Natural killer cells (NK) were introduced into the capillary fluid flow circulation of MIVO, mimicking blood capillary flow. Flow cytometry analysis demonstrated tumor-mediated extravasation of DNAM1+ NK cells and their infiltration into the 3D tumor models, leading to an initial reduction of tumor cell viability. A combined drug safety and efficacy assay was developed by fluidically connecting 3D ovarian cancer tissues with a hepatic cellular model: The efficacy of cisplatin (i.e., decrease of the SKOV-3 viability) and its hepatotoxicity (i.e., death of Hep-G2 cells) were measured simultaneously, opening the possibility to drastically reduce the time and costs of preclinical assays. A linear decay of Hep-G2 and SKOV-3 cell viability was observed with increasing cisplatin concentration after 48 h of treatment. The 3D ovarian cancer model demonstrated higher drug resistance than the 2D model and reduced efficacy against the 3D ovarian cancer tissue, and hepatotoxicity was observed in the MIVO compared with the single organ model (0.5X of EC50 and 2X of LD_{50}). In conclusion, this highly predictive, functional, and relevant human tissue model, through adoption of the MIVO device, can be efficiently employed as a drug screening platform for both pharmacological treatments and cell-based therapies, and for basic research purposes.

Paola Occhetta, BiomimX, Milan, Italy, presented "*Beating organs-on-chip: from single functional tissues to multiorgan models*". The uBeat® platform integrates human cells, 3D culture, hydrogel scaffold, and mechanical stimulus and allows the real-time determination of some electrical parameters. Cardiomyocytes self-organize, express a more differentiated phenotype, and can beat synchronously in response to mechanical stimuli, representing a suitable model for drug screening. The uGut platform combines Caco2 and HT29 MTX cells and applies stretching forces to better mimic gut peristalsis. Reconstructed organs are more viable, differentiated, and functional. Different organs-on-chips (liver and heart, liver and tumors, dorsal root ganglia and muscle) can be constructed to reproduce systemic communication between organs in health and disease.

Fiorella Scagnoli, San Raffaele Hospital, Milan, Italy, presented the talk "*Establishment of 3D multi-organ system in a milli-fluidic bioreactor to study the chronic lymphocytic leukemia (CLL)* in vitro". CLL is characterized by an accumulation of monoclonal B lymphocytes in the bloodstream, bone marrow (BM), and peripheral lymphoid tissues. Based on expertise in 3D BM models (Barbaglio et al., 2021) and in establishing a long-term 3D bioprinted CLL culture (Sbrana et al., 2021), humanized and miniaturized 3D lymph node (LN) and BM models were recreated to mimic *in vivo* CLL cell behavior and achieve the cellular complexity of the two tissues. Lymphatic fibroblasts and endothelial cells for the LN, and mesenchymal stem cells differentiated into osteoblasts, adipocytes, and endothelial cells for the BM were grown in a versatile, gelatine-based scaffold. In parallel to the static condition, the IVtech flow-perfusion bioreactor was utilized to generate the biophysical forces approximating the *in vivo* physiologic context. CLL cells will be introduced to study their phenotype and behavior in the presence or absence of a therapeutic agent.

CellTox Days 2022 highlighted research and development towards achieving more reliable and relevant *in vitro* models able to reproduce physiology as well as pathologies. To date, conventional animal tests are still indispensable for human risk assessment although they are considered inadequate to provide the needed safety data in a timely and cost-effective manner since it may not always be readily translatable to humans. New approach methodologies offer many advantages as they allow higher efficiency, are less expensive, and are focused on human cells, but they still need optimization to fulfill scientific and regulatory expectations.

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