Meeting Report

3Replacement Winter School – Out of the barriers: *In vitro* models in toxicology

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The first 3Replacement Winter School entitled *Out of the barriers:* In vitro *models in toxicology*, focused on epithelial barriers and their applications in toxicology, held in Brescia on November 27, 2017, was hosted and organized by the *Istituto Zooprofilattico Sperimentale della Lombardia e dell'Emilia Romagna, Centro di Referenza per i Metodi Alternativi, Benessere e Cura degli Animali da Laboratorio* in collaboration with the Italian Association of *in vitro* Toxicology (CELLTOX) and the Italian Platform on Alternative Methods (IPAM).

Dr **Isabella De Angelis** (*ISS, Rome*), with a lecture on *In vitro barriers: Relevance and innovation*, gave an overview on epithelial barriers, their characteristics and future applications. Epithelial cells border most organ luminal compartments and external body surfaces, establishing a selective barrier that regulates the exchange of solutes and nutrients between different compartments. Epithelia are also important as a line of defense against the intrusion of pathogens, although several bacteria have developed strategies to overcome them, e. g., by interfering with trans-cellular transport processes or tight junctions. On the other hand, epithelial barriers represent the major obstacle for targeted drug delivery. To fulfill their highly specialized functions, epithelial cells assume a polarized architecture. Narrow junctions (*zonula occludens, tight junctions*) seal off the adjacent cells, regulating substance passage through the cellular lamina and allowing the maintenance of cellular homeostasis. Determination of absorption through epithelial barriers is the first step for toxicokinetic studies. Many advanced *in vitro* models of epithelial barriers are available (skin, corneal, buccal, nasal, lung, intestinal, renal, blood-brain, and placental). They provide well-defined systems to investigate basic properties of epithelial cells, as well as mechanisms of barrier absorption and impairment after chemical treatments or in specific diseases. In order to obtain well-differentiated epithelial cell layers *in vitro*, it is necessary to reconstitute their natural microenvironment as far as possible. This is generally achieved by culturing the cells on Transwell filters that maintain two separate compartments, the apical compartment facing the luminal environment and the basolateral one facing the sub-epithelial tissue. Current developments of *in vitro* models of epithelial barriers include: i) use of pluripotent human stem cells; ii) development of specialized models including multiple different cell types; iii) 3D models; iv) dynamic cultures (on-a-chip).

Elena Dell'Ambra (*IDI-IRCSS, Rome*) gave a presentation on *In vitro skin models and their applications*. Skin is the barrier between the body and the environment and carries out many essential functions for human survival. Skin displays a passive barrier function, such as support and protection against mechanical stress and dehydration, and various resident cell types carry out active barrier functions, such as protection against pathogens, chemicals and UV radiation, as well as thermoregulation and sensation. Alteration of these barrier functions generates several skin pathologies. Skin equivalents, composed of dermal fibroblasts embedded in a biological matrix and keratinocyte layers, have been developed to reproduce key structural and functional properties as therapeutic tools for large and chronic skin lesions. The integration of skin equivalents with other skin cell types (e.g., immune and vascular cells) allows the study of mechanisms controlling skin homeostasis and its disruption as models of skin diseases. Recent breakthroughs in tissue engineering, such as the development of induced pluripotent stem cell (iPSC) technology, microfluidic platforms and 3D bioprinting, herald the next generation of skin equivalents that may represent more reliable skin disease models and promising tools for drug screening. Indeed, there is a need for human skin models for drug development since animal models are not representative of the structure and physiology of the human skin.

In vitro models of the intestinal barrier were presented by **Yula Sambuy** of *CREA Food and Nutrition Research Centre, Rome*. The intestinal mucosa represents an important site for the study of potentially toxic substances introduced as dietary contaminants or as pharmaceutical agents. In fact, direct toxic effects to intestinal cells not only harm the organ itself, but have effects at the systemic level by breaking down the barrier and allowing potentially toxic substances to reach other organs or to produce immune reactions and inflammatory responses. The best available model of the human small intestinal mucosa is the Caco-2 cell line, obtained from a human colon adenocarcinoma, but capable of differentiating in culture on filter inserts, reproducing the polarized organization of the intestinal epithelium and expressing several morphological and functional features of the mature enterocyte. Suitable culture procedures and different applications of this cell line in toxicology were described, including applications in nanotoxicology and in co-culture with other cell types. Recent 3-D gut models from human donors were also described. The future of human intestinal barrier models will probably come from the field of iPSC, which are used to derive several differentiated cells, and tissues using normal, non-cancerous tissue. However, iPSC-derived intestinal barrier models are still in development and it will take some time before they are used for toxicological studies.

Maurizio Gualtieri (*ENEA, Bologna*), with the presentation *Air blood barrier and particles: The guardian of a constrained*

path, showed the *in vitro* respiratory epithelial barrier and its possible application in toxicology. Airborne particulate matter is among the more relevant environmental causes of increased morbidity and mortality. Depending on the aerodynamic diameter, four classes of particulate matter (PM) are usually defined: PM_{10} (diameter ≤ 10 µm), $PM_{2.5}$, PM_1 and $PM_{0.1}$ or UFP. Finer particles ($PM_{2.5}$ and below) reach the deep lungs and therefore represent a potential health risk. The integrity of the air blood barrier (ABB) is fundamental to control and avoid the translocation of inhaled particles. Several *in vitro* models have contributed to the understanding of the toxicological effects of particles on lung epithelia, e.g., translocation of UFP through the ABB and mechanisms to counteract particle-induced damage. New *in vitro* models mimic the interaction between air-dispersed particulates and cellular membranes more closely. Air liquid interface systems now also allow evaluation of effects at concentrations relevant for human environmental exposure.

Development of *in vitro* models to accurately predict *in vivo* drug toxicity is one of the greatest challenges of the pharmaceutical industry today as **Hassan Rashidi** (*University of Edinburgh, Scotland*) presented in *A novel three-dimensional hepato-spheres model for long-term evaluation of drug toxicity*. Freshly isolated human adult hepatocytes are considered to be the gold standard to evaluate human drug metabolism and safety *in vitro*. However, primary hepatocyte scarcity, cell cycle arrest and the rapid loss of liver-phenotype post isolation are major limitations. Immortalized and hepatoma cell lines have therefore been employed as potential alternatives, however their poor functionality, karyotypic instability and higher tolerance to toxicological insult limit their widespread application. Human embryonic and induced pluripotent stem cells can be differentiated into hepatocyte-like cells (HLCs) *in vitro*. Although HLCs can be derived efficiently from pluripotent stem cells under conventional monolayer protocols, they exhibit fetal features and have a transient phenotype, which limits their applications. A protocol was developed to derive functional HLCs under three-dimensional (3D) conditions. The 3D HLCs downregulate expression of the fetal marker alpha-fetoprotein by day 30 of differentiation and exhibit a stable phenotype for over 365 days *in vitro*. More importantly, the cells remain metabolically active and drug-inducible during the culture period, providing an *in vitro* platform to better evaluate long-term effects of new lead compounds.

Nanoparticles and the *in vitro* placental barrier were the topics of **Luisa Campagnolo** (*Università di Tor Vergata, Roma*) with *In vitro model for developmental toxicity study*. Nanotechnology is an expanding field in which NP are developed for several purposes and their characteristics can be exploited, e.g., to improve medical devices; however, evaluation of the potential risks of NP on public health is propaedeutic to their use. Evidence exists that NP can reach and cross biological barriers, including the placenta. In order to screen for the ability of NP to breach the placental barrier and to interfere with embryonic development, a cost-effective, rapid *in vitro* screening system is being developed using trophoblast stem cell (TSC) lines derived from mouse blastocysts. TSCs are multipotent cells that can differentiate into large, multinucleated syncytia, resembling syncytiotrophoblast cells of the placenta. TSCs are differentiated on transwell inserts and mouse embryonic stem (mES) cell derived embryoid-bodies (EB) are cultured in the lower compartment. The EBs recapitulate *in vitro* embryonic development (issuing derivatives of all three germ layers), and are routinely used to test chemical compounds for embryotoxicity. Gene expression analysis can be performed for genes expressed by the endoderm, mesoderm and ectoderm of EBs cultured with or without NPs, in the presence or absence of the simulated barrier.

Another perspective was introduced by **Francesca Caloni** (*Università degli Studi di Milano-DIMEVET*), who introduced and described many epithelial barriers of human and animal origin in her presentation entitled *In vitro 3D species-specific models*. The best *in vitro* toxicological approach should be chosen on the basis of the mechanism of action under investigation and should consider the concept of species-specificity. *In vitro* animal barriers include bovine mammary epithelial cells (BME-UV), dog skin equivalent 3D, porcine alveolar epithelial cells (pAEpC), and porcine small epithelial cells (IPEC-J2). Human *in vitro* barriers, such as Caco-2 cells, human skin model and epithelial respiratory barrier, may also be considered for risk assessment in veterinary toxicology. Animal cell-based *in vitro* models may also be useful in evaluating human effects, e.g., IPEC-J2-MDR1. Relevance, robustness and reproducibility are the 3Rs for development and application in toxicology of a reliable model.

A fruitful general discussion with an active audience, composed of students, PhD students and researchers, concluded the meeting.

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