Meeting Report

# From 3Rs to 3D: *In Vitro* Alternative Models for Replacement

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On February 21, 2018, a third meeting was held at the University of Agricultural Sciences and Veterinary Medicine (USAMV) in Cluj-Napoca, Romania with representatives of the Romanian Center for Alternative Test Methods (ROCAM), the Italian Platform on Alternative Methods (IPAM) and the Italian Association of *in vitro* Toxicology (CELLTOX). The main objective of the meeting was to promote alternative testing models in research and education and to present different *in vitro* models to be used for xenobiotic testing.

Prof. **Francesca Caloni** (Università degli Studi di Milano-DIMEVET), emphasised, during her presentation entitled "Innovative 3D *in vitro* alternative models in Veterinary Science", that new *in vitro* models for safety assessment are urgently needed to evaluate risks related to the environment, and human and animal health in accordance with the application of the 3Rs (Directive 2010/63 EU). She also presented *in vitro* predictive models, from 2D to epithelial barriers, that could be used in veterinary medicine, especially veterinary toxicology, such as porcine IPEC-J2 and IPEC-J2 MDR1s intestinal epithelial cells, bovine mammary epithelial cells cultured on inserts, stem cell based *in vitro* models, and feline hepatic organoids. These species-specific models could be promising as stand-alone models or as part of a testing strategy.

Dr **Teresa Coccini** (ICS Maugeri – IRCCS Pavia) gave a presentation by video conference call entitled "Species-specific cell-based model: human CNS co-culture system platform for toxicity screening". An *in vitro* model was implemented using a Transwell system combining human neuronal cells (SH-SY5Y cell line) and astrocytes (D384 cell line) to investigate protection of neurons by astrocytes and *vice versa*. The model was applied to test the cytotoxicity of different neurotoxic agents, such as methyl mercury, iron oxide nanoparticles and methylglyoxal. All tested compounds affected mitochondrial activity and cell morphology in both mono-culture and co-culture conditions. However, astrocytes, when cultured together with neurons, diminished the cytotoxic effects of all tested agents observed when neurons were cultured alone, and astrocytes were more resilient in the presence of neurons. This human CNS co-culture model can be an effective tool to investigate toxic effects of different compounds on the CNS, including new and emerging contaminants (e.g., nanomaterials, toxins); it provides a more representative human *in vivo*-like tissue and it provides species-specific information on the mechanisms of neurotoxicants, avoiding many of the uncertainties and problems that are inherent to interspecies extrapolations.

Dr **Alessia Bertero** (Università degli Studi di Milano – DIMEVET) presented by video conference call a project entitled "Emerging mycotoxins: Toxicological evaluation with *in vitro* model for endocrine disruptor". Mycotoxins are low molecular weight secondary metabolites produced by a large variety of moulds that can contaminate foods and feeds. A high prevalence of the so-called "emerging" mycotoxins has been recently detected using new technology. These mycotoxins frequently occur in combination with traditional mycotoxins such as zearalenone, a known endocrine disruptor, and many others. The aim of the project is to evaluate the endocrine disruptor effects of emerging mycotoxins and their possible interactions with traditional mycotoxins by exposing bovine granulosa cells to emerging mycotoxins alone and combinations with traditional mycotoxoins.

**Cristian T. Matea** and **Teodora Mocan** from the Regional Institute of Gastroenterology and Hepatology Cluj-Napoca, Department of Nanomedicine and University of Medicine and Pharmacy Cluj-Napoca, Romania, spoke on "Alternative approaches for biological testing in nanomedicine". One key aspect of nanoparticle-based therapy and diagnostics is the proper characterization of the pure and functionalized nanomaterial. The advantages of using dark-field hyper-spectral microscopy as a tool for nanoparticle characterization and to determine the distribution of nanoparticles inside cells were presented and the use of atomic force microscopy force-distance curves to determine the rigidity of cells co-incubated with nanoparticles was explained. Concepts for constructing biological testing platforms, such as *in vitro* blood exposure to nanoparticles, erythrocyte toxicity assessment and examples of *ex vivo* platforms using human liver or pancreas explants were introduced. Also, examples of dynamic *in vitro* platforms, such as chemotaxis, migration and adhesion assays using cell lines were presented and primary macrophage cell culture with nanoparticle activation followed by skin Langerhans cell targeting was discussed as a preliminary testing option for anticancer effects. The use of simulated body fluid for standardized testing of digestive stability and behaviour of bioparticles was presented, as well as available methods for the assessment of particulate absorption along the different digestive segments. Practical aspects for each platform, including reproducibility issues, milestones, positive aspects and limitations were also discussed. All concepts presented demonstrated the need for considering alternative methods of biological testing, requiring both innovation and standardization.

Dr **Dumitrita Rugina** (University of Agricultural Sciences and Veterinary Medicine, Cluj-Napoca) presented a study entitled "Novel microcapsules as carriers for resveratrol delivery inside living cells". Diabetic retinopathy (DR) causes visual impairment, and may cause blinding, due to damage of the retina. Current therapies developed for DR include anti-vascular endothelial growth factor (VEGF) therapies, focal laser therapy and steroids. Numerous natural compounds have been tested as potential protectors of the retina, either through their antioxidant potential or by inhibition of angiogenesis. However, the beneficial effect of natural compounds is limited by their low bioavailability. In recent years, general attention has been placed on the use of resveratrol (RV) (3,5,4'-trihydroxystilbene) for prevention or complementary therapy for eye diseases. RV is known as a phytoalexin with low water solubility and reduced stability. A desirable strategy to enhance the bioavailability of RV is to increase its solubility and stability by developing a delivery system to be used in local therapies. Retinal pigmented epithelial cells (RPE) can be used as a model of DR. RPE cells are first adapted to low glucose culture medium (5 mM) and then exposed to high concentrations of glucose (30-60 mM) in order to mimic hyperglycaemic conditions. RV were loaded in polyelectrolyte multilayer microcapsules (PEMs), which were delivered into RPE cells. RV-PEMs were obtained by a layer-by-layer assembly approach and were characterized by spectroscopic and microscopic methods. The amount of RV that reached the target site was assessed by HPLC-ESI-MS analysis. Confocal and scanning electron microscopy proved that 2-4 RV-PEMs could be internalized in D407 cells, having no cytotoxic effect. RV-PEMs were able to suppress the expression of VEGF protein, indicating that RV-PEMs could be used as anti-VEGF agents, as an alternative to the current therapy. The present strategy could also be used in laser therapy, where the release could be triggered by the laser light reaching the retina cells.

Dr **Cristina Coman** (University of Agricultural Sciences and Veterinary Medicine, Cluj-Napoca) gave a presentation entitled "Raman mapping: a tool for imaging nano- and microstructures after cellular uptake". Raman mapping is a non-invasive, label-free technique with high chemical specificity and high potential to become a leading method in biological and biomedical applications. High spatial resolution imaging of biological samples, individual cells and tissues is possible, due to an excitation wavelength in the visible and near-infrared range. The technique provides chemical coupled with spatial information. The advantages, limitations and potential applications of the technique were discussed. The presentation focused on using Raman mapping as a tool for the detection of cellular uptake and intracellular distribution imaging of nano- and microstructures. In particular, the possibility of using the technique for *in vitro* studies for monitoring the internalization of gold nanoparticles in adenocarcinomic human alveolar basal epithelial cells (A549) was discussed. Raman spectra of cells need to be analysed by principal component analysis (PCA) and the end result consists of images of the sample, so-called false colour Raman maps, containing highly precise structural and chemical information and showing cytoplasmic internalization of the nanoparticles. Also, the use of Raman mapping for imaging of polymeric microstructures after cellular uptake in human retinal epithelial (D407) cells was discussed, with the conclusion that the microstructures are engulfed by cells and found to be located in the cell cytoplasm.

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