Meeting Report

Replacement, Reduction, Refinement: 3 days for 3Rs

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The international IPAM webinar "Replacement, Reduction, Refinement: 3 days for 3Rs" was organized by the Italian Platform on Alternative Methods[1] (IPAM) during February 2022. The webinar was organized in 3 separate sessions, each addressing one R. More than 100 participants from different EU countries attended the whole webinar series.

The first day, chaired by **Francesca Caloni** (Università degli Studi di Milano, Italy) and **Francesco Nevelli** (Merck, Italy), was focused on Replacement.

After a brief presentation of the program by **Isabella De Angelis** (Istituto Superiore di Sanità – ISS, Italy), IPAM president, the first talk by **Fabrizio Lecce** (Merck, Italy) was titled *Monocyte Activation Test. An* in vitro*, faster and ethically driven alternative to detect pyrogens*. Pyrogens are a heterogeneous group of microbial and non-microbial contaminants producing adverse effects in patients ranging from fever to death. The most widely known pyrogens are the lipopolysaccharides, also known as endotoxins, of Gram-negative bacteria. Testing parenteral pharmaceuticals for the presence of pyrogens is a regulatory requirement to ensure product safety. Methods to detect pyrogen contaminants include the bacterial endotoxin test (BET) and the recombinant Factor C (rFC), which both detect endotoxins only, while the *in vivo* rabbit pyrogen test (RPT) and the monocyte activation test (MAT) recognize the entire pyrogen spectrum. Merck products were evaluated by MAT for the low endotoxin recovery (LER) effect, which is the inability to detect endotoxin contaminations due to masking effects caused by chelators or detergents used in buffer formulations (Reich et al., 2016). Four Merck products showed such an LER effect. Their masking activity was investigated, and experimental protocols were modified to overcome the LER effect with the aim to establish a more ethical and efficient pyrogen testing approach based on the *in vitro* MAT compared to the traditional approach consisting of BET coupled with RPT.

Marisa Meloni (VitroScreen, Italy) described the experience of VitroScreen gained through a project addressing thyroid endocrine disruptors (EDs). EDs can interfere with the endocrine system, causing adverse health effects in an intact organism, in its progeny, or in sub-populations. The OECD scoping document on thyroid hormone signaling modulators (OECD, 2014) has identified eight potential mechanisms of thyroid system disruption. Since 2019, as a member of the EU-NETVAL network, VitroScreen has been involved in a validation study, coordinated by EURL-ECVAM, on *in vitro* methods for the detection of thyroid disruptors. VitroScreen was engaged to assess human TRα and TRβ reporter gene assays developed and commercialized as a kit by Indigo Biosciences (USA). The first part of the study focused on the in-house testing of the method and on the assessment of its reliability and performance, while the next step will assess the relevance of the method using coded test items.

Silvia Letasiova (MatTek In Vitro Life Science Laboratories, Slovakia) gave a lecture entitled In vitro *skin irritation test for medical device extracts using EpiDerm tissue model: The way from* in vivo *to* in vitro *testing*. Assessment of dermal irritation is an essential component of the safety evaluation of medical devices. Reconstructed human epidermis (RhE) models have replaced rabbit skin irritation testing for neat chemicals (OECD TG 439). However, medical device extracts are diluted solutions with low irritation potential. Therefore, the validated RhE methods were modified to reflect the needs of ISO 10993. A protocol employing RhE EpiDerm was optimized using known irritating chemicals and polymers spiked into extraction solvents (Casas et al., 2013). Seventeen laboratories were trained in the use of the protocol in preparation for assay validation, and all produced data with almost 100% agreement of predictions for the selected reference materials (Kandárová et al., 2018a). Moreover, several medical device benchmark materials (5 irritants and 2 vehicles) were evaluated in controlled human patch testing (4 h and 18 h) and using the EpiDerm *in vitro* skin irritation protocol, and results were compared to existing rabbit skin irritation test data (Kandárová et al., 2018b). An international round robin validation study was conducted to confirm the ability of the RhE models to correctly predict the intra-cutaneous irritation of extracts from medical devices. EpiDerm tissues were able to correctly identify all irritant polymer samples in saline or in sesame oil or in both solvent extracts (De Jong et al., 2018). The use of the reconstructed tissue models to replace the rabbit intra-cutaneous test is implemented into the ISO 10993 standards used to evaluate medical device biocompatibility.

The second day, chaired by **Cristina Failla** (Istituto Dermopatico dell'Immacolata, IDI-IRCCS, Italy) and **Augusto Vitale** (Istituto Superiore di Sanità – ISS, Italy) was dedicated to Reduction.

[1] www.ipamitalia.org

Alessandro Giuliani (Istituto Superiore di Sanità – ISS, Italy) introduced the concept of Reduction from a wide theoretical perspective. He argued that the behavior of biological systems emerges from the correlation of different players at all biological and organization levels from molecules to ecosystems (Uversky and Giuliani, 2021). This implies that there is no single privileged level of explanation (prediction) for biologically relevant endpoints. To get meaningful information from experiments from gene expression to ecosystem modelling, including studies on animals, we must rely on correlations among different observables. The analysis of the correlation structure among observables allows to single out the "latent structures" of the studied data set that in turn mirror the system functioning. The computation of such correlations asks for multiple measurements (in space, as diverse experimental variables, and in time) to be executed on the same animal. This allows a drastic reduction of the number of animals and presents a more realistic picture of the investigated phenomena.

Laura Ricceri (Istituto Superiore di Sanità – ISS, Italy) explained that for researchers to deal with Reduction still means being out of their comfort zone. However, there is an intimate relationship between study design and the Reduction principle: Only after careful sample size estimation can Reduction be applied and the total number of required subjects be computed. Applying Reduction thus currently means to apply the proper design and statistical tools to determine the lowest number of animals necessary to perform statistically robust and reproducible experiments. *In vivo* researchers should be aware that reproducibility issues have been recently raised also for *in vitro* studies. Wide opportunities for further improvements remain: Focussed training, specialized guidelines, and editorial policies can synergically operate to make the animal research community more aware and willing to implement the Reduction concept (NPQIP, 2019).

In his talk, **Luca Bonini** (University of Parma, Italy) explained that brain stimulation techniques are crucial to probe causality in the brain-behavior relationship and to modulate brain activity in clinical conditions such as, for example, deep brain stimulation to treat Parkinson's disease or closed-loop neuroprosthetic to restore motor skills in amputees and subjects with spinal cord lesions. However, conventional brain stimulation techniques are invasive and limited by technical problems. Among non-invasive brain stimulation approaches, focused ultrasound stimulation (FUS) allows reversible neuromodulation of individual or even multiple deep brain structures at submillimeter resolution in the same subject. Novel FUS approaches will therefore allow to further reduce and refine basic research with non-human primates and increase their validity by adopting within-subject designs. FUS techniques may also contribute to replace non-human primate use with healthy human subjects and selected patient populations in at least some brain stimulation studies.

The third day, coordinated by **Stefano Lorenzetti** (Istituto Superiore di Sanità – ISS, Italy) and **Augusto Vitale** (Istituto Superiore di Sanità – ISS, Italy), was focused on Refinement.

Alessandra Taglioni (Consiglio Nazionale delle Ricerche – CNR, Italy) introduced the positive reinforcement training (PRT) technique, which is a refinement procedure that can be considered a form of social enrichment in the form of the relationship with the trainer. The technique decreases stress and stress-related variability because the animals know what to do and what will happen. Furthermore, PRT reduces some abnormal behaviors, increases the safety of procedures by reducing the level of aggression and fear in experimental subjects, usually in response to constriction measures, and increases the frequency of affiliative behavior between animals and trainers. PRT exploits the ability of animals to associate a reward, usually a preferred food, with a behavior. Positive reinforcement needs to immediately follow the desired behavior (such as presenting a part of the body for inspection or moving from one cage to another). A clicker can be used as a bridge, i.e., a secondary reinforcer that helps the subject understand which behavior is desired even before the primary reinforcer is delivered. The sound of a clicker is a marker that usually ends the behavior. A trainer will need to understand the character and predisposition of the subject, learn to create a protocol suitable for their scientific purposes, and break a complex behavior down into small steps.

Laura Calvillo (Università degli Studi di Milano, Italy) explained that pain and stress dramatically affect animal physiology, leading to suffering and to bias in the results. It is therefore essential to quantify the level of discomfort experienced by animals housed in animal facilities and when subjected to experimental procedures. Three simple methods are available: Manual behavior analysis (MBA), the Mouse/Rat Grimace Scale (MGS/RGS), and nesting, which allow a reproducible quantification of welfare by means of a digital camera and human operator analysis. Infrared thermography and HomeCageScan can be added for automatic detection and analysis of the animal physiological state. Moreover, by observing animals during biomedical research, it is possible to refine the performed procedures (e.g., improving analgesia and detecting typical signs of suffering) without recruiting further animals (reduction) and to quantify the beneficial effect of non-aversive handling.

Finally, **Johnny Roughan** (Newcastle University) described methods that provide a starting point towards gaining surrogate evidence as to the "affective" nature of animal pain. He summarized methods that have been used by the Pain and Animals Welfare Science (PAWS) group at Newcastle University towards more effective determination of pain severity; both post-surgical and in nociceptive assays that are commonly used to evaluate analgesic potency. These were: naturalistic behavior assessment, conditional drug discrimination, conditioned place preference, analgesic self-administration, and facial expression analysis. Concerning the last methodology, mice experiencing pain display changes in facial expression (known as grimacing) following exposure to a variety of different painful challenges. Grimace scales have been developed for a range of different species including rats, rabbits, and horses and can provide a useful and rapid method both for pain detection and in determining whether an-

algesic treatments are effective. The PAWS group has used this technique to establish that pain occurs in mice with lung cancer (Miller and Roughan, 2022), and following surgery and methods of mouse identification (Roughan and Sevenoaks, 2019).

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