State of the Art on Alternative Methods to Animal Testing from an Industrial Point of View: Ready for Regulation?

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Summary

Despite changing attitudes towards animal testing and current legislation to protect experimental animals, the rate of animal experiments seems to have changed little in recent years. On May 15-16, 2013, the In Vitro Testing Industrial Platform (IVTIP) held an open meeting to discuss the state of the art in alternative methods, how companies have, can and will need to adapt and what drives and hinders regulatory acceptance and use. Several important points arose from the meeting. First, industry and regulatory bodies should not wait for complete suites of alternative tests to become available, but should begin working with methods available right now (e.g., mining of existing animal data to direct future studies, implementation of alternative tests wherever scientifically valid rather than continuing to rely on animal tests) in non-animal and animal integrated strategies to reduce the numbers of animals tested. Second, sharing of information (communication), harmonization and standardization (coordination), commitment and collaboration are all required to improve the quality and speed of validation, acceptance and implementation of tests. Finally, how alternative methods can be used in research and development before formal implementation in regulations should be considered. Here we present the conclusions on what can be done already and suggest some solutions and strategies for the future.

Keywords: non-animal testing, legislation, regulation, in vitro modeling, harmonization, standardization

Highlights

- In spite of partial replacement of methods in testing strategies, in no area can animal testing yet be completely replaced.
- Substantial progress is needed in the development of methods to replace, reduce and refine animal experiments (the 3Rs approach).
- The emphasis on mechanisms and modes of action will be crucial to minimize animal studies in toxicological evaluation.
- All stakeholders must not only promote the use of alternative tests, but also take responsibility to find pragmatic ways to change.
- Sharing of information, harmonization, standardization and collaboration will help to improve the quality of tests and speed of validation.

1 Introduction

Despite changing attitudes towards animal testing and striking advances in technology, substantial progress has been made in the development of alternative methods to achieve replacement, reduction and refinement of animal experiments (the 3Rs approach). Although the rate of animal testing varies between regions, little seems to have changed in recent years in Europe (EC, 2010). Legislation is in place that encourages the phasing out of animal testing (EU, 2010), yet some companies seem reluctant to move away from proven animal tests to new testing strategies. For the cosmetics industry animal testing and marketing of animal-tested products is now banned, but no validated assays are available to replace tests for most classic toxicological endpoints and kinetic data. Therefore, alternative approaches are needed to ensure safety, but the change needs to be much more strongly supported by all stakeholders.

On May 15-16, 2013, the In Vitro Testing Industrial Platform (IVTIP) held an open meeting to discuss the state of the art in alternative methods, how companies have, can and should adapt and what helps and hinders regulatory validation. The meeting included perspectives from industry, regulators, contract research organizations and societies for the humane treatment of animals. Several key themes became clear. Here we present the conclusions on what can be done now and suggest some solutions and strategies for the future.

2 Current status of legislation

On July 11, 2013, the European Cosmetics Regulation (EC/1223/2009) fully replaced Council Directive 76/768/EEC, which was put in place in 1976 to ensure the free circulation of safe cosmetic products in the European internal market. The seventh amendment of the Directive in 2003 introduced the phasing out of animal testing and, eventually, marketing of products that have involved animal testing (CEC, 2003), for which the final deadline passed on March 11, 2013. Despite the lead-up period and the extension of animal testing for some complex endpoints, the European Commission acknowledges that the full replacement of animal testing by alternative methods for toxicokinetics, skin sensitization, repeated dose testing and reproductive toxicity is not yet possible (EC, 2013a). It has, however, decided to go ahead with the ban for several reasons: It was feared that further postponement of the ban or allowance of applications for dispensations on testing would not reflect the political preferences of the European Parliament and could "*diminish determination to swiftly develop alternative test methods. Past experience demonstrates clearly that animal testing provisions in the cosmetics legislation have been a key accelerator in relation to the development of alternative methods and have sent a strong signal far beyond the cosmetics sector and far beyond Europe*" (EC, 2013a). An impact assessment (EC, 2013b) showed that stakeholder opinions diverged on the degree of impact related to the ban coming into force as planned. Also, where animal tests were phased out in 2009 for which no alternatives were available, no major negative impacts have been seen so far (EC, 2013a). In all industries dealing with compounds and ingredients, legislation has been in place since 2010 (2010/63/EU) that requires companies to prioritize non-animal methods where they are available (EU, 2010). Bridging the gap between knowledge and legislative commitments by the innovative development of predictive models by *in vitro* assessment of pathways and mechanisms is, therefore, an urgent requirement.

3 Alternative methods and approaches

3.1 Mechanisms and modes of action

Although the study of mechanistic data is not new, the emphasis on mechanisms and modes of action in toxicological evaluation is a notable shift and will be crucial to minimization of animal studies. Knowledge of modes of action will provide information on key events from the molecular to the organism levels that can be applied to assessment of exposure risks and population responses. Several challenges, however, will need to be faced. First, it is unlikely that any single animal test will be replaced by a single alternative test, and multiple approaches will need to be applied to achieve the same results. Thus, rather than thinking about the quality of individual tests, researchers will have to start designing testing strategies that include several tests, which will take more planning and require more advanced validation assessments. Also, the risk that results will be obtained by chance due to increasing the number of tests, and ways to counteract those effects will need to be taken into account at the study design stage (e.g., sample size, significance threshold, etc.). Owing to the increase in the number of tests and the potential for high-throughput analyses, the volume of data will also be greatly increased. How to store, organize, present and interpret larger amounts of information in a transparent and useful way will need to be further explored and harmonized (Hardy et al., 2012). Not least of the challenges is how to open up industry and regulators to the changes in testing strategies. Modes of action as part of the 3Rs strategy are relatively newly acknowledged (Zuang et al., 2013) and there is a lot to learn. So far, the greatest amount of experience with alternative methods has been gained in the pharmaceutical industry. However, recently, the Safety Evaluation Ultimately Replacing Animal Testing (SEURAT-1), a Seventh Framework Programme project, jointly financed by the European Commission and the cosmetics industry, has been set up to assess the role of mechanistic and mode-of-action research in toxicology.

Adverse outcome pathways (AOPs) represent the sequential progression of events from exposure to outcome (Ankley et al., 2010). They are conceptual constructs of linkages between a direct molecular initiating event and adverse outcomes at different levels of biological organization and are, therefore, highly relevant to risk assessment. Information can be classified at the molecular, macro-molecular, cellular, organ, organism and populations levels. The aim is to identify ways in which normal processes are altered by exposure to stressors and whether or not these changes result in harmful outcomes. In theory the number of ways in which cells function should be finite, although the pathways by which alterations occur could be infinite. Although extrapolation of the results to the human population will take years, mapping of these toxic effects has begun to build the human toxome. The pathways concept represents a paradigm shift from the traditional safety evaluation of identification of a hazard, risk characterization and exposure assessment to predictive science (Leist et al., 2008).

Alternative methods for testing of skin sensitization, carcinogenicity, and repeated-dose and reproductive toxicities have also begun to be developed as part of broader testing strategies. Most of the key stages in skin sensitization AOPs can now be tested by laboratory *in vitro* tests with skin models, peptide reactivity assays, activation of innate response, epidermal equivalent potency testing and activation of dendritic cells. Some of these test methods have entered regulatory-driven validation studies and acceptance is foreseen in the near future. Additionally, where tests might have been limited to a few doses in animals, owing to cost, resource and legislative constraints, *in vitro* assays allow a wide range of doses to be tested. This should yield much more highly relevant information on (low) dose-response effects where in animal tests the minimum internal dose that

causes an effect might be irrelevantly high compared with that required in humans (Crump, 2011).

The Organisation for Economic Co-operation and Development (OECD) has suggested that AOPs be recorded according to a standardized notation (OECD, 2013). It purports that the three basic elements for AOPs are molecular-initiating events, which describe how the chemical interacts with the initial biomolecule; intermediate events; and adverse outcomes, which should be specific and well-defined. Key issues in building AOPs are where to start (which pathway), how and where to limit them, how to assess and identify completeness and robustness, how to identify which uses will be appropriate and the validation to humans. The building of pathways can start from any of the three basic elements but, although adverse outcomes can result from multiple molecular initial events and vice versa, each AOP should be limited to a single molecular initial event and one adverse outcome. Information from different levels of biological organizations, however, can be integrated into a pathway. The OECD also provides guidance on the minimum conditions for evidence (Bradford-Hill criteria; Hill, 1965), annotation of the pathway, quality assessments, scope and how to make quantitative linkages.

AOPs already help to direct testing by enabling the linkage of events to outcomes or testing of predicted relations assessed *in vitro*. Thus they are likely to have multiple applications that can reduce the need for animal testing (Tab. 1). To help prediction, AOPs will need to show key actions that link events and how pathways intersect and interact and, therefore, creation of predictive paradigms is complex. This will hopefully become easier as more AOPs are created, developed and assessed, although information from a lot of AOPs will be needed. Thus an additional challenge will be improvement of the tools for and approaches to storage and access of AOPs to avoid duplication and ensure standardization.

3.2 Emerging technologies and techniques

Traditional toxicology animal testing relies heavily on dose tests followed by the detection and pathological evaluation of manifested toxic lesions. The so-called omics technologies, bioinformatics, systems biology and computation biology (Altenburger et al., 2012; Collings and Vaidya, 2008; Panagiotou and Taboureau, 2012) enable high-throughput analysis of treatment-related changes at the molecular level and, therefore, might provide a means for predicting toxicity before classic toxicological endpoints are seen. The pillars of modern toxicology could be considered as organotypic cultures including "human-on-chip", omics (high-throughput studies, bioinformatics, bioengineering), pathways of toxicity (human toxome) and integrated testing strategies.

In the USA, the National Toxicology Program (http://ntp.niehs.nih.gov/) recognized that the technological advances in molecular biology and computer science offered an opportunity to use *in vitro* biochemical-based and cell-based assays and non-rodent animal models for toxicological testing. These assays allow for high throughput at a much reduced cost. In some assays, many thousands of chemicals can be tested simultaneously in days. The National Toxicology Program is collaborating with other federal organizations, the NIH Chemical Genomics Center and the National Center for Computational Toxicology, the Environmental Protection Agency and the Food and Drug Administration, to develop the Tox21 project. It aims to develop, validate and translate innovative, high-throughput chemical screening methods to characterize key steps in toxicity pathways, identify mechanisms of action for further investigation and develop predictive models for *in vivo* biological response. Currently, more than 10,000 compounds (8,100 of which are unique substances) are being analyzed by a variety of automated methods. This enables high-throughput analysis to be undertaken day and night, which yields results much faster than could ever be achieved by human teams. At the meeting, it was purported that this approach will be the future of industry testing if the AOPs can be translated or linked to human diseases.

3.3 Use of existing resources

Irrespective of whether animal testing remains legal in certain industries or whether validated non-animal models are available, a huge amount of data have been collected from years of animal testing. The phasing out of animal experiments does not mean that these data become invalid. Rather, they comprise a valuable resource that should be perused carefully to help guide the development of non-animal alternative methods and prioritize future areas of study (Ekin, 2006; Hardy et al., 2012; Rebholz-Schuhmann et al., 2012; Schrage et al., 2011), particularly while alternative methods are awaiting approval. *In vitro* tests should not be validated by comparison with animal studies, which sometimes have limited predictive value compared with the human situation, and new thinking about the validity of new approaches must be adopted to ensure innovative approaches are approved.

Tab. 1: Roles for adverse outcome pathways in reducing the need for animal testing

Current and near-term uses of adverse outcome pathways:
- Inform chemical categories and structure activity relationships
- Identify hazards
- Prioritize chemicals for further assessment
- Support and enable interpretation of existing and new information
- Contribute to development of integrated testing strategies that maximize useful information gained from minimum testing

Possible future roles:
- Prioritization of a wider range chemicals for assessment
- Identification of key events for which non-animal tests can be developed to facilitate mechanism-based, non-animal chemical assessment
- Creation of predictive toxicological assessments with low uncertainty and high human relevance
- Eventually lead to the replacement of animal testing

Mining of existing data obtained with technologies, such as metabolomics and transcriptomics, systems biology and bioinformatics, as well as new experiments, are helping to systematically organize knowledge of what happens from exposure to a toxicant to effects at the molecular, macromolecular, organism and population levels. This approach has played an important part in the Tox21 initiative. Existing data were provided with many of the compounds that were contributed to the project and have yielded important information to guide studies.

3.4 The relationship between industry and regulators

A substantial proportion of Member States do not yet adhere to 2010/63/EU, which requires prioritization of non-animal tests over animal tests whenever possible. That IVTIP is concerned about compliance with this requirement was clearly represented at the meeting. The reasons underlying poor adherence to legislation are manifold and are seen at multiple levels. A key phrase in the legislation is "*Member States shall ensure that a procedure is not carried out if another method or testing strategy for obtaining the result sought, not entailing the use of a live animal, is recognized under the legislation of the Union*." Exactly what is classed as recognized, however, is unclear and interpretations seem to vary widely. Where to find clarifying information also seems unclear. Thus, the number of regulatory bodies and companies making substantial efforts to adopt alternative methods to animal testing might be reduced.

To temper this uncertainty, the relationship between industry and regulators needs to be strengthened. Guidance on what tests are already available and prompt notice of any new tests (validated or non-validated) and how to incorporate them into testing strategies is needed at international and national levels and across sectors. Any such guidance must be relevant to optimize human safety. To make the guidance relevant, regulators need as much information as possible and, therefore, companies must show they understand the tests they apply and also present data in a clear but concise way. Ethics committees and national reference laboratories should receive up-to-date information frequently and promptly. Sharing of information, collaboration and harmonization between regulatory authorities, companies and experts need to become the norms. Education will be crucial to achieving change. Young scientists in universities and even in schools need to be aware of animal use and inspired to explore alternative methods.

3.5 Validation time frames

The time to validation can take years and the processes can be complicated, confusing and frustrating, which might deter companies from developing novel alternative methods. In Europe, the risk assessment process is done at two levels: one at the European Commission level and one at industry level. Companies must create dossiers on all the substances they use. These are passed to the responsible parties in the company (safety assessors) and the European Commission (Directorate General for Health & Consumers) who must ensure the information is made clear to the public. The European Commission assigns substances to annexes – forbidden substances, restricted substances, colorants, preservatives and ultraviolet filters. Thus, the product information for all ingredients must be comprehensive to enable assignment to the correct annexes. Industry and the European Commission both use the same safety evaluation strategy: hazard identification, dose response for risk characterization and exposure assessment. Where this was traditionally done with *in vivo* tests, industry is now encouraged to take a 3Rs approach (replace and/or refine wherever possible to reduce the numbers of animals tested) and to review existing data from previous *in vivo* tests, from analogues and read-across studies. A major drawback that could be preventing greater degrees of innovation and of applicability, however, is that *in vitro* data generated for one industry frequently cannot be transferred for use by another. In the European Union, legislation for different industries (e.g., chemicals, food additives, cosmetics, pharmaceuticals and detergents) exists in parallel with little communication or collaboration. Additionally, having to work within OECD regulation guidelines removes the flexibility needed to accommodate product-related modifications. Thus, tests are duplicated for each industry and resubmitted for validation. At the same time, however, some "horizontal" legislation applies across all industries in all European Union countries, such as that on animal experimentation. To satisfy all requirements in this legislative structure can become incredibly difficult and requires a lot of excess time and effort. Increased communication and collaboration between industries and regulators would be an important step towards improving the validation process.

Application of methods after validation is another area of concern. The European Coalition to End Animal Experiments (ECEAE), a Europe-wide alliance, reported in the meeting that it has found that implementation of alternative methods is frequently held up after validation, which prevents quick and broad uptake. The alliance suggests that post-validation stages need to be taken into account during the process to speed and smooth the implementation of new tests. It summarizes the validation and post-validation stages as assessment, decision, acceptance, policing and transparency (ADAPT). The first hurdle is the decision about whether a novel approach needs to be tested before submission for validation (assessment) and is confused by a lack of clarity about which regulatory authority is the most suitable to assess a new approach and make the final decision that an alternative method is valid (decision). Reliance on international acceptance, for instance through further assessment by OECD, can substantially delay regional implementation and some methods are initially approved but face legal hold ups to implementation (acceptance). As suggested above, once methods are validated and accepted, it must be ensured that companies use them in preference to pre-existing animal tests (policing). Finally, the regulatory requirements and processes must be simplified and the frequency and effectiveness of collaborations and communication must increase (transparency).

4 Innovations and potential solutions

Although it became clear at the meeting that there are many hindrances to moving towards alternative methods, many positive points were raised about innovative thinking and possible ways to overcome hurdles that might be widely applicable. These ranged from ways to use existing data and how to record, organize and store new data, potential ways to increase the dissemination of informa-

tion, improve information sharing and increase collaboration for models that have been developed to expand the range of substances that may be tested *in vitro* and state-of-the-art methods to analyze high throughputs of data and ways to address wider issues.

The Sneller van Innovatie naar Mens (SLIM), or Faster from Innovation to Man, project is an ongoing group project in the Netherlands that involves the government, pharmaceutical, food and other companies, academic institutions and various other partners. The objective is to identify good practices for smarter and faster development, acceptance (including regulatory) and implementation of 3Rs methods. The aim is to investigate how 3Rs approaches will fit better into current practices and processes, barriers to and drivers of regulatory acceptance and how industrial and regulatory needs can drive future goals. Four fields of research areas are being assessed: reproductive toxicity, food allergy, carcinogenicity and exposure barriers. These represent complicated areas of safety assessment for which the development of alternative methods is difficult (EFSA, 2009). Some of the non-animal methods that have proven helpful so far are mining of existing data and creation of comprehensive, widely accessible databases, use of *in vivo* repeated-dose toxicity studies to move away from 2-year rat carcinogenicity studies, use of human cell assays, use of high-throughput studies, and *in vitro* and *in silico* modeling of bioavailability. However, multiple factors – drivers as well as barriers – at different levels (individual, organization, institution, authority and society) influence innovation and need to be taken into account.

Repeatedly during the meeting, the issues came up of access to and dissemination of information. Rather than to tweak current approaches, a fundamental shift to "open source" information was suggested. This concept is widely understood by the IT community, has been utilized for around the past 30 years and has led to many innovations on varying scales, from small pieces of software to whole computer operating systems. To be deemed truly open source, a product and its intellectual property must be publically available, acceptable and readable, must not be subject to copyright or intellectual property protection, and elements may be altered by multiple developers to make the product fit for purpose. In any industry there are, of course, risks in making properties openly available. A particular issue in industries bound by safety analysis is whether regulation can be upheld if a product is made openly available after validation. That should not mean, however, that open source is wholly inapplicable to validated tests in Europe. It was suggested that exposure of products in the pre-validation stages could lead to optimized methods through the feedback of experts. In turn, the honed final product might be quicker and easier for regulators to approve. An adapted form of open source was suggested, in which tissue models and test performance data, but not the "source code" behind them, are made publically available with the right granted for others to produce and use those models in any way. This approach might help companies in restrictive shipping areas or small companies to access robust models by gaining the ability to make them on site. Of course, any tests and models would have to be developed to the highest standards and minimum requirements would need to be put in place and adherence proven. Any negative issues might, however, be outweighed by the benefits of a swift, wide dissemination of information (De Wever et al., submitted)

It was well recognized by meeting participants that the range of novel *in vitro* models and other methods will need to be broad to replace animal tests. Two innovative examples were presented. First was a three-dimensional airway epithelium model that is developed from nasal, bronchial, or tracheal epithelial cells that differentiate into all the relevant upper airway cells. This model is accompanied by a novel method to enable testing of non-water compatible substances. The substance is mixed with dextran in serial solid dilutions, compressed and the resulting tablet is applied to the epithelial model surface. Exposure is homogeneous across the surface of the model and the reactions are as they would be *in vivo* because of direct contact with the epithelium. Several endpoints may, therefore, be tested simultaneously. Currently, this model is used as part of an integrated testing strategy, the results are donor specific and limited numbers of cultures can be generated per donor (100-500 inserts), although a large pool of donors is overcoming this problem (35,000 inserts). Although not yet stand-alone, this model illustrates how difficult substances can be dealt with in custom made models that have a long shelf life (approx. one year).

The next approach discussed was an imaging software package for which protocols may be adapted to suit the client's needs. Assessments can be fully automated and can improve accuracy, reproducibility, rate and cost-effectiveness of analysis. The software enables high-throughput analyses of individual elements within a sample. For instance, from an original red-green-blue image of a stained slide from the epithelial airway model, mucin and nuclei can be viewed separately (Fig. 1). Additionally, target regions can by highlighted by delineation or zoning (block coloring of the different elements in one image). This tool can help with the study of efficacy and toxicity and is widely applicable across the chemical, cosmetic, pharmaceutical and other industries.

Less innovative but just as important solutions are harmonization and standardization across countries, areas of regulation and industries. Such changes would greatly improve the regulatory process and the sharing of information and would reduce test duplication, effort and costs. In terms of test development, innovation will rank much higher than competition. For instance, as animal tests become phased out, it will be difficult to introduce competition for two similar tests. Unless there is a major innovative difference between them, the customer will be inclined to buy the cheaper one. Harmonization, therefore, could save not only end users' but also developers' time and money and encourage innovation and sharing of information. Again, a new approach is needed that moves away from company and product specificity. Standardization of terminology, test materials generation and delivery, methods of information storage and access will encourage integration of all stakeholders and international acceptance, which will in turn help with regulatory validation.

Finally, where alternative tests have been validated but the required tools are not yet readily available, some companies have designed their own instruments and made them available to other companies. Such activity has been undertaken by BASF with the development of the BCOP Opacitometer Kit BASF – OP3.0 (http://on.basf.com/1sNtuOZ)

5 Conclusions

Partial replacement or refinement of methods as part of comprehensive testing strategies in order to reduce the number of animal tests has been possible, and some methods have been validated. In no area, however, can animal testing yet be completely replaced. Despite the time involved in development and validation, an overwhelming message to come out of the meeting was that it is better to try to create alternative methods that better predict the human situation than not to try at all. Mode-of-action and mechanistic studies to improve understanding of the pathways that lead to harm will play an important part in the required paradigm shift. Industry needs to start building strategies, initially on the basis of what tools are available right now, to help direct efficacy and safety questions, to adopt properly the alternative methods available and to move towards as little animal testing as possible. While there are animal protection laws in place that make animal tests a last resort, many hindrances, such as costs, shipping, expertise and so on mean that widespread implementation of sufficient alternative methods to replace them is slower than necessary. All stakeholders – industry, regulators, government, academia, animal welfare organizations and consumers – need, therefore, to not only promote the use of alternative tests, but also to co-ordinate to find pragmatic ways to help more companies implement them. The key to success will be an integrated approach that bridges the distance between science, policy, legislation and education to enable knowledge transfer. Companies will need to be persuaded that gains, such as improved prediction of effects, cost effectiveness and so on, can be made in the long run by not using animal models. In the broader arena, guidance on scientific issues related to safety should be sought from independent bodies, such as the Scientific Committee on Consumer Safety (SCCS), forums should be created for information sharing and exchange of ideas and solutions (safe harbor concept), and stakeholders should consider joining together to start dialogues about hindrances and other issues in an attempt to speed resolutions. Proactivity and collaboration to design and validate widely applicable, cost-effect alternative methods are urgently needed on all fronts.

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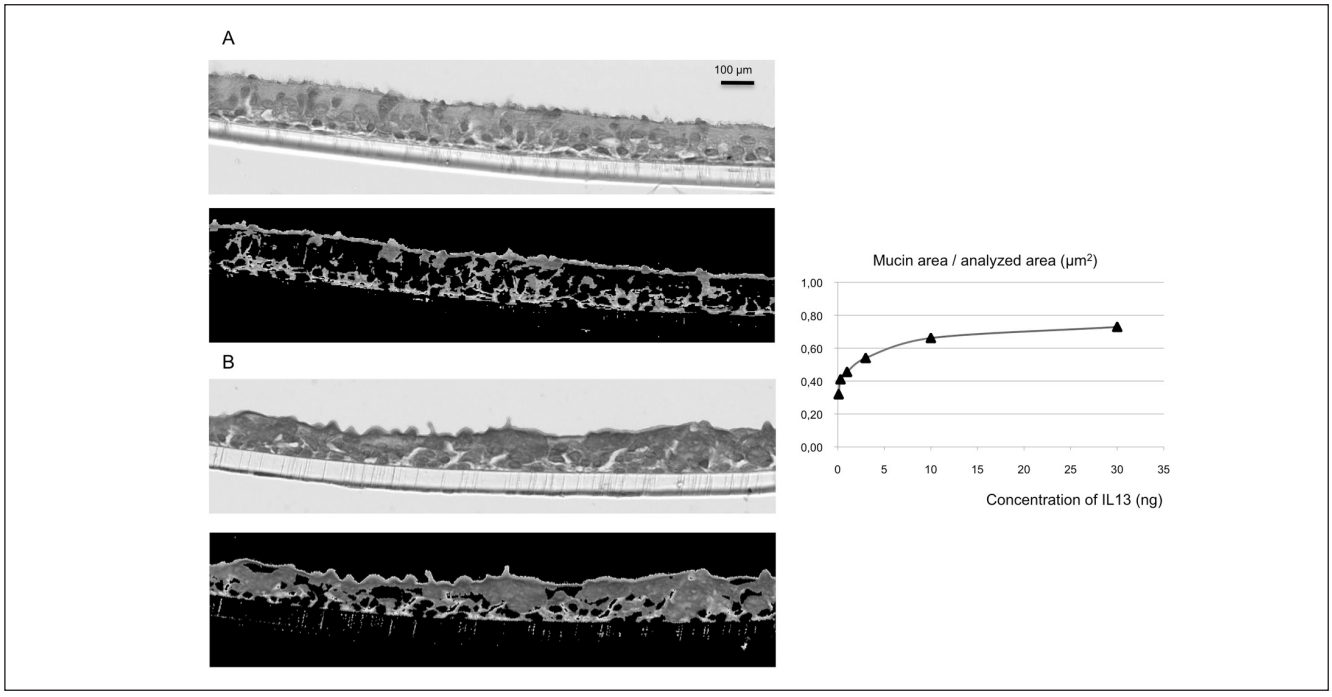

Fig. 1: Use of image-processing software to quantify mucin in goblet cells in a three-dimensional model of human bronchial epithelium

Mucin secretion was induced by an increased concentration gradient of IL-13 and sections from the model were stained with alcian blue. Discrimination processes were applied with the software to create images of the mucin component. Images of section (A) unaltered MucilAir (top) and after processing to show only the mucin component at 0.1 ng/ml (bottom) and (B) unaltered (top) and after processing to show only the mucin component at 30.0 ng/ml (bottom). All images ©BIOCELLVIA, Marseilles, France.

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Conflicts of interest

The authors declare they have no conflicts of interest.

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Developing Microphysiological Systems for Use as Regulatory Tools – Challenges and Opportunities

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In the last few years, scientists have made important progress in developing systems using human cells to test the effects of drugs and other substances. These systems have the potential to improve toxicity testing beyond currently available tools. The innovative new tools, which are known as microsystems, microphysiological systems, or organs on a chip, can aid in the development of medical products so that toxicity may be identified earlier in product development. This may lower costs and speed new treatments to patients. Experts believe that these systems may eventually enable scientists to test more environmental compounds more efficiently.

On May 10, 2013 more than 220 scientists in academia, industry and regulatory agencies met in person and virtually to discuss the essential elements needed to develop these systems for use as regulatory tools, as well as pathways to their qualification as regulatory tools. The one-day workshop was co-sponsored by the Food and Drug Administration, National Institutes of Health, National Institute for Environmental Health Sciences, National Center for Advancing Translational Science, Environmental Protection Agency, Johns Hopkins School of Public Health's Center for Alternatives to Animal Testing and the International Consortium for Innovation and Quality in Pharmaceutical Development.

Jesse L. Goodman, the chief scientist of the Food and Drug Administration (FDA), opened the workshop by predicting that microphysiological systems may impact both the specificity of toxicology and how it is modeled. "We have many compounds and interventions that we have to reject now that if we can improve specificity we may not have to reject." The tools may help illuminate the reasons for toxicity, as well as how genetics may come into play, he says. The tools also hold promise for furthering the study of disease models, Goodman said. "Being able to have 3-dimensional complex models using human cells offers tremendous potential."

Donald E. Ingber, the director of the Wyss Institute for Biologically Inspired Engineering at Harvard University described the potential of microphysiological systems from the academic perspective. In 2010 Ingber and colleagues produced what they call a lung-on-a-chip and described it in *Science* (Huh et al., 2010). The chip recreates the alveolar-capillary interface, one of the major functional units in the lung and the site where oxygen enters the body. This same interface is where aerosol-based drugs are delivered, where some cancers can metastasize (Huh et al., 2012), and it is a major site where pneumonias develop, among other things. The lung-on-a-chip is the size of a computer memory stick, Ingber said. It was inspired in part by recent advances in microfluidics, which involves use of computer microfabrication techniques to construct networks of hollow channels that can control and manipulate fluids at sub-milliliter scales to take advantage of changes in how the fluids behave at these microscopic dimensions. The researchers were able to use the lung on a chip to mimic common lung functions and generate predictions about previously unknown functions that were confirmed in studies with whole mouse lungs. For example, they showed that exposure to airborne particulates in the form of colloidal silica nanoparticles can create an inflammatory response in the lung on a chip, and they discovered that the cyclic mechanical strain of breathing accentuates the toxic and inflammatory responses. "Breathing alone does not achieve this, and the nanoparticles alone do not do it. Only together does this happen," Ingber explained. The Wyss researchers have also successfully mimicked pulmonary edema,

a deadly condition in which the lungs fill with fluid and blood clots (Huh et al., 2012).

The pharmaceutical industry's view of microphysiological systems was provided by James L. Stevens, a distinguished research fellow at Lilly Research Laboratories, the research and development arm of Eli Lilly and Company, one of the world's largest pharmaceutical corporations. He shared his thoughts on how microphysiological systems can help improve safety assessment by avoiding toxicity, predicting target organ toxicity, and managing risks in clinical trials. He said that Lilly tends to use microphysiological systems "on the back side" after a negative outcome has been brought to light to replicate the pharmacology and biology to see if the problems can be avoided or managed. For example, Lilly scientists have used human cardiomyocyte heart muscle cells derived from induced pluripotent stem (iPS) cells to identify pharmacology-based impacts on cardiovascular function. He says that when pharmacologists know a target organ, microphysiological systems can also focus screening.

Douglas Throckmorton, deputy director for regulatory programs for the Food and Drug Administration's (FDA) Center for Drug Evaluation and Research, discussed microphysiological systems from a regulatory perspective. He reminded the audience that new drug success rates are not as good as they could be (Kola and Landis, 2004; Arrowsmith et al., 2011). To address these problems, FDA needs to support the development of predictive physiological biomarkers, Throckmorton continued. The process through which biomarkers are currently generated, which tends to be on a case-by-case basis driven by drug manufacturers' needs with a slow movement towards general use as scientific experience accumulates, is inefficient. In this context, Throckmorton perceives that microphysiological systems offer "profound opportunities." In addition to being non-animal-based, they can be highly efficient in terms of the number of products that can be screened. They also have the potential to be integrative, in that they offer the potential to answer more than one question about a drug's effects.

Thomas Hartung, the Doerenkamp-Zbinden Professor and Chair for Evidence-based Toxicology at Johns Hopkins University, gave a talk on the topic of Good Cell Culture Practices (GCCP) and quality control. He told the audience that he believes the human on a chip microphysiology approach can help overcome important shortcomings associated with using *in vitro* models. The fact that they have the potential to mimic the differentiation of organs is a positive, as is their potential to supply oxygen to the cultured cells. Another plus is that microphysiological systems are likely to overcome problems associated with the tumor origin of many cell lines as they mainly make use of induced pluripotent stem cells. 3-dimensional cultures offer some improvements over 2-D cultures, Hartung continued. These include increased cell survival and differentiation. The 3-D cultures also allow for increased cell-to-cell interaction, and they do a better job of reproducing the complexity of human organs. However, scientists don't yet have a plan to stress the human-on-a-chip cells continuously, so they are likely to be as "bored" as conventional *in vitro* cells, which Hartung sees as a key reason for dedifferentiation *in vitro*. There is as yet no way to mimic metabolism or stimulate defense, either. Thus far there also are no plans for introducing analytics to determine the fate of test compounds in culture. Validating organs-on-a-chip presents a new challenge, Hartung told the audience. There's a dramatic difference between a model and a test, he explained. Just as liver cells are a model, microphysiological systems are models, not tests, he continued. A given test is defined by its purpose, Hartung stated. It is defined by a very precise protocol. For example, hepatocytes can be cultured in different ways to produce an endless number of different tests depending on the intended goal and how the parameters are set up. While animal models have been mostly accepted based on their "face validity" because they use a healthy living organism, microphysiological systems are essentially devices with a variety of different elements. As models, they must be defined by precise protocols based on the phenomenological similarity to the organisms and/or current scientific understanding.

Kyle Kolaja of Cellular Dynamics International (CDI), a company that manufactures human iPS tissues, made a case for why stem cell-derived tissues can help researchers develop microphysiological systems. Stem cell-derived cells are likely the most ideal format available for generating the cells and tissues used in microphysiological systems, Kolaja said. From the perspective of toxicology, the limitations of primary cell culture have hindered the potential of replacing animal and human experiments, Kolaja continued. The challenges associated with making the shift include consistent access to primary human and animal cells, variability due to how cells can be isolated, and the degeneration of the *in vivo* phenotype once in culture. By addressing these issues, stem cell-derived tissues can also help further the development of the 3-dimensional models needed to produce microphysiological systems. A key advantage of stem cells results from how they can be derived via genetic engineering, Kolaja told the audience. "The iPSC field has moved past the early days of integrated viruses," he said. "Now methods that do not require integration in the genome are used predominantly to reprogram cells. Small amounts of peripheral blood or other tissue can be used as starting material. The iPS cells are grown in defined media and on well characterized matrices, two improvements that have helped provide consistency to stem cell culture," he explained.

Danilo Tagle, the associate director for special initiatives of the National Institutes of Health's National Center for Advancing Translational Science, chaired a session highlighting efforts to build a representative microphysiological organ system.

D. Lansing Taylor, the director of the University of Pittsburgh's Drug Discovery Institute, talked about his institute's collaboration with the Massachusetts General Hospital's Center for Engineering in Medicine to create a liver on a chip. Specifically, their 3-D system replicates the liver's acinus and sinusoids. These are the areas where nutrients, fats, toxins and bacteria that enter the liver via venous blood from the gut are processed. The 3-D liver chip that Taylor's group has created has a grooved design intended to mimic the hepatic chords in the acinus. The channels created by the microgrooves represent sinusoids. Each sinusoid groove contains all the essential cell types found in the liver, including Kupffer cells, stellate cells, endothelial cells and hepatocytes. The chip uses microfluidics to control the environ-

ment and the flow rates of medium continuously bathing the liver chip. The group's goals for the 3-D liver platform include reducing drug attrition rates by recapitulating the human liver acinus physiology and making the optimal measurements to characterize it. A key element of this is building a predictive database, Taylor explained. "We also want to use all of the data we're collecting for a variety of systems biology modeling tasks," he said.

Donald E. Ingber gave his second talk of the day on the efforts of his group at Harvard's Wyss Institute for Biologically Inspired Engineering to build representative microphysiological organ systems. He focused mainly on his group's successes in constructing models of the human gastrointestinal system, including the microbiome, but he also discussed research underway toward recreating a human kidney proximal tubule, a small airway, and bone marrow on chips. The Wyss researchers have produced what they call a peristaltic human gut on a chip, Ingber said. They started with the lung organ and modified it to mimic the human intestine with its microbiome. They made the organ higher and wider and set it up to produce a trickling type of a flow similar to the one in the human gut. Rather than exerting breathing motions, it was engineered to have the cyclic deformations associated with the wave-like contractions of peristalsis. They used human CaCo-2 colon cancer cells, which are known to not be well-differentiated and in existing static culture systems appear more like skin cells than gut cells. However, after just three days of experiencing the flow and strain in the artificial gut on a chip, they began to look columnar, like gut cells. Over time, they spontaneously reorganized to form villi, which are the cell-lined finger-like projections that are normally found lining the human gut. Just like the villi in the human intestine, these structures have tight junctions and are covered with mucus. The structures also include the crypts containing proliferative cells found in the human gut, which include four different types of differentiated epithelial cells (absorptive, mucus-secretory (Goblet), enteroendocrine, and Paneth) that take characteristic positions similar to those observed in the living human small intestine. Ingber's group has also used primary human intestinal epithelial cells to recreate the same structure with the gut on the chip, as recently described in articles in *Lab on a Chip* (Kim et al., 2012) and *Integrative Biology* (Kim and Ingber, 2013). The fact that colon tumor cells work so well to reproduce the intestinal physiology and even produce mucous suggests that the cells are "rebootable," especially since CaCo-2 cells are known normally not to produce mucous in static cultures. Transcriptome profiling of the gut epithelium revealed that the expression of about 10% of 22,203 human genes was significantly altered in mechanically active environments including flow and/or strain. Just trickling flow can alter the phenotype of these cells, and trickling flow plus cyclic strain change them in an entirely different way, Ingber said.

Jonathan Himmelfarb, the director of the University of Washington's Kidney Research Institute, talked about his group's efforts to create a kidney on a chip. The formula for how the kidneys clear drugs and toxins was first laid out almost 100 years ago. The rate of filtration and the ability to reabsorb through the kidney's nephron and tubules is well-understood and both can be easily modeled clinically. "But to this day we cannot effectively model tubular secretion of drugs and toxins," Himmelfarb said. To the pharmaceutical companies, tubular secretion remains a black box in terms of understanding either if any compound is secreted or the extent to which it is going to be secreted, he explained. The functional unit that Himmelfarb's group set out to model includes the vasculature of the peritubular capillaries with the pericytes, which communicate with the blood vessels' endothelial cells, and the proximal tubule. This unit is critical to kidney toxicity and how the kidney eliminates drugs. The proximal convoluted tubule cells are full of mitochondria and are highly active metabolically. "The work of the proximal tubule is all about transport, whether it's reab-sorption or secretion. It's really the factory in the kidney for the transport of solutes," he said. Its functions also include the generation of ammonia from glutamine, or ammoniagenesis, and the 1-α hydroxylation of vitamin D. It is also the cell in the kidney that is most subject to injury because it is so metabolically active and is exposed to such high concentrations of the kidney filtrate, Himmelfarb said. By including three types of cells, the proximal tubules, the pericytes, and the microvascular epithelium, Himmelfarb said his group's tubular interstitium on a chip should be able to effectively model the kidney's secretory process. The chip they are designing includes a parallel tubule and a parallel microvessel.

The final session of the conference focused on the integration of microphysiological organ systems. **Robert Kavlock**, the Deputy Assistant Administrator for Science within the Environmental Protection Agency's (EPA) Office of Research and Development, told the audience that he was "absolutely amazed by the progress" in creating the microphysiological organ systems that the previous speakers described. Both scientists in the pharmaceutical research area and in the environmental health research area are motivated by the new technologies' ability to "allow us to get inside the black box" between the exposure to a chemical and the responses that can be detected by animal testing, Kavlock said. For EPA, the driver is the need to collect toxicity data. In the long run "how we integrate all of these systems together and how we share knowledge and share information… will help us to move forward – whether you're interested in drug development or whether you're interested in the effect of chemicals in the environment," he said.

Melvin Andersen, the Charles E. Hamner Distinguished Fellow at the Hamner Institute, discussed what the new systems do that current systems are incapable of achieving. He also asked the audience to consider where the successes achieved at this early stage of development will lead us. The new platform may enable scientists to test more environmental compounds more efficiently and it may also help speed the testing of new drugs and biological products for which human efficacy studies are neither ethical nor feasible (products which fall under the FDA's animal rule which allows approval based on the proof of efficacy in animals) by allowing studies to be conducted on a limited human platform. In order for the platforms to have value, scientists need to be confident that they are capturing the likely toxic responses, Andersen stressed. A key issue that Andersen said he would like to see addressed is how microphysiological systems can be used mechanistically with human tissue aggregates to get a better understanding of modes and mechanisms of action. He pointed out that "no matter how we hook these tissues together, they don't

add up to a human on a chip. Each one of them lacks critical components to be a full tissue, but it still can be tremendously useful," he said. If the goal is to improve evaluation of the effects of chemical exposures, Andersen suggested that it may make sense to consider more carefully which tissue systems should be included in the first test systems. Rather than rushing to combine all of the organ systems to produce a "truncated human on a chip," Andersen pointed out that the collaborators may do well to show that a more limited platform can faithfully represent expected tissue exposures in an intact organism.

Suzanne Fitzpatrick, Senior Science Advisor of the FDA's Office of the Commissioner's Office of the Chief Scientist closed the meeting. "In putting together this program with the FDA and CAAT, we wanted to create a community of people who were all working toward a common purpose," she said. She credited the National Research Council's *Toxicity Testing in the 21st Century* report for helping to catalyze the creation of such a community. "To move innovation, we really need a whole community of people… to look at these new toxicology models," she concluded.

A more detailed summary of the workshop is available at: http://www.altex-edition.org

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3Rs and New Frontiers in Laboratory Techniques

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On May 8, 2014, a meeting on advanced laboratory techniques was held by the Italian Reference Center for Alternative Methods, Welfare and Care of Laboratory Animals. The meeting, hosted by the Istituto Zooprofilattico Sperimentale della Lombardia e Emilia Romagna, was opened by Dr **Stefano Cinotti** (ISZLER) and chaired by **Francesca Caloni**, University of Milan and **Gianni Dal Negro**, GlaxoSmithKline.

The first speaker, Dr **Marlies Halder** (JRC, Ispra, Italy), provided a comprehensive overview on methods for quality control of vaccines. Halder showed how such methods for new vaccines are almost exclusively based on *in vitro* tests, while for old vaccines, which have until now been tested using animals, the "consistency approach" is increasingly being embraced, reducing animal use. Halder expressed the hope that Directive

2010/63/EU will speed up the validation process of alternative methods in the context of vaccines. **Julie Holder** (GSK, UK) gave a presentation on induced pluripotent stem (iPS) cells in regenerative medicine with a particular focus on cardiomyocytes and provided a state of the art overview of methods currently available for 3D iPS cultures in heart research. Dr **George Loizou**, (Computational Toxicology Team, Mathematical Sciences Unit, Health & Safety Laboratory Buxton, UK), shared present and future perspectives in computational modelling applied to toxicology. Loizou emphasized the critical role that human data will play on developing animal free approaches in toxicology by applying the P4 (Predictive, Preventive, Personalized, Participatory) medicine approach. **Robin Williams** (Centre for Biomedical Science, Royal Holloway, University of London, UK) presented the social amoeba *Dictyostelium* as a screening model in early drug development. In particular, Williams presented some data on the possible application of this eukaryotic cell model to predict bitter taste of oral formulations and replace the rat pica model. **Paolo Bazzicalupo** (Institute of Biosciences and Bioresources, Italian National Research Council, Naples, Italy) presented the nematode *Caenorhabditis elegans* as a model organism for pharmaco-toxicological studies and screening purposes (Kaletta and Hengartner, 2006) and for various human pathologies, in particular neurodegenerative diseases. *C. elegans* is suitable to address fundamental biological questions, Bazzicalupo said, for the following reasons: i) ease of experimental manipulations, ii) anatomy and development described in great detail (it is transparent) and iii) powerful molecular genetics approaches (>70% of C. elegans genes have homologs in other organisms and >55% of C. elegans genes have a significant human match, including many genes involved in human diseases). In 2006 the US National Toxicology Program started a project to develop medium- throughput toxicity screens using *C. elegans*.

The afternoon session, chaired by **Isabella De Angelis**, ISS and **Giovanna Lazzari**, Avantea, was opened by Dr **Thomas Keller** (GSK, UK), who spoke on human relevant high content screening platforms based on organ-functionalities-on-a-chip. Tissue chips are engineered microsystems that represent units of human organs, such as lung, liver and heart, modeling both structure and function. This technology aims to improve the reliability of drug development and toxicology screening. In particular, Dr Keller described a human gut-on-a-chip model that mimics the complex structure and physiology of living intestine using human intestinal epithelial cells (Caco-2) maintained under a low rate fluid flow. These conditions accelerate intestinal epithelial cell differentiation, promote formation of 3D villi-like structures and increase intestinal barrier function; the addition of cyclic mechanical strain that mimics normal peristaltic motions, further enhances these responses. Moreover, once differentiated within the gut-on-a-chip device, the intestinal epithelium can support growth of microbial flora that normally lives within the human intestine (as *Lactobacillus rhamnosus* GG) without compromising its viability (Kim et al., 2012). The human peristaltic gut-on-a-chip may greatly improve the study of the mechanic regulation of intestinal function, as well as host-microbe symbiosis and evolution. In his second presentation, Dr **Loizou**, presented an innovative *in silico* PBPK model designed to predict tissue concentration and biologically effective dose. PBPK models can support extrapolation across dose-routes, different dose and exposure scenarios and between species; inter-individual variability and sensitive subpopulations also can be represented. PBPK modelling also is successfully used in reverse dosimetry, or dose reconstruction, approaches, where environmental exposure is calculated based on biomarker measurements from blood or urine samples. *In vitro* data, derived from both cell culture or toxicogenomic assays, may be also utilized. The reverse dosimetry approach has great potential as a tool for risk assessment.

In the round table discussion, participants shared their opinions on relative replacement, distinct from absolute replacement in which animals are not used at all. Despite different interpretations, there seemed to be a consensus that *in vivo* and *in vitro* activities are inherently connected and complementary. Participants agreed that relative replacement represents the best compromise at this time, while looking forward to putting an end to our reliance on animals in research activities.

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National Workshop/CME on Alternatives to Animal Experimentation in Medical Science Education

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Introduction

The concept of alternatives to animal experimentation is relatively new to India (Akbarsha et al., 2013). The ethical issues regarding use of animals in life sciences and biomedical sciences gained attention with the work done by People for Animals (PfA), People for Ethical Treatment of Animals (PeTA) and Mahatma Gandhi-Doerenkamp Center (MGDC) established by Doerenkamp-Zbinden Foundation (Switzerland), and regulations and guidelines have been brought up by the regulatory authorities such as ICMR (Indian Council of Medical Research), CPCSEA (Committee for the Purpose of Control and Super-vision of Experiments on Animals), MCI (Medical Council of India), PCI (Pharmacy Council of India), MoEF (Ministry of Environment and Forests, Government of India) and UGC (University Grants Commission) during the past 10-15 years (Badyal et al., 2009; Shehnaz et al., 2012, 2011).

The Department of Pharmacology, Jawaharlal Nehru Medical College (JNMC), Aligarh Muslim University (AMU), Aligarh, has been active in this field since the early days of these developments (Rai and Singh, 2006). In 2006 the department started a separate laboratory known as *Alternatives to Animal Experimentation Lab* for training medical students in alternatives in pharmacology (Ranganatha and Kuppast, 2012) using software from various sources. Since then, the number of animals used for teaching and practice has been drastically reduced. The third issue of a dedicated manual on *Alternatives to Animal Experiments* by Dr Syed Z. Rahman and Dr Mohamed Tariq Salman was published this year.

With a view to further propagate the concept of alternatives among the scientific community of India utilizing the experience of the department, a national workshop-cum-CME (continuing medical education) on *Alternatives to Animal Experimentation* was organized at JNMC on February 24-25, 2014. The program was supported with grants from MCI and AMU. Fifty-two participants (postgraduates in the fields of pharmacology, physiology, anatomy, Unani medicine, etc.) received hands-on-training on the latest software available to replace the use of animals in teaching fundamental concepts of medical sciences, especially physiology and pharmacology. The lectures and training were provided by persons from different parts of India.

Inauguration

Prof. **Shahjahan Bano** (Dean, Faculty of Medicine, JNMC, AMU), in her introductory remarks, said that the workshop would help students to improve their skills in teaching and research by using simulators and software and without harming animals. Prof. Tariq Mansoor (Principal and Chief Medical Superintendent, JNMC) reminded listeners of their moral obligation to care for animals and to avoid causing pain and distress to them. Dr **Chaitanya Koduri** (Science Policy Advisor, PeTA India) lauded JNMC's efforts regarding non-animal methods in teaching and learning which fulfil the legal obligation to protect animals as prescribed in the Constitution of India. Dr **Syed Z. Rahman** (Organizing Secretary) said that the main objective of organizing the workshop was to provide participants with an opportunity to explore the expanding possibilities of alternatives to animal experimentation, to train and instill confidence in them for use of alternatives and to encourage them to adopt these alternatives in teaching and training. A souvenir-cum-scientific proceedings (edited by Dr Umme Aiman) was released on this occasion (Rahman, 2014).

Keynote addresses

In the first keynote address Prof. **Krishan Chandra Singhal**, a leading pharmacologist, summarized how animals played crucial roles in development of not only drugs but also surgical procedures. He, however, emphasized that these potential benefits could be attained without subjecting experimental animals to any form of suffering. While speaking about alternatives to animal experiments, he advocated that a committee of knowledgeable scientists and educationists should share a common platform for discussing the pros and cons of alternatives to animal experimentation, assessing input required for educational set-ups and finding funds for the purpose.

In the second keynote address Prof. **Mohammad A. Akbarsha**, Director and Chair, MGDC, explained that many animal experiments are not relevant to humans owing to species differences, particularly pertaining to phase I and phase II metabolic enzymes. He elaborated on the evolution of *in vitro* and *in silico* approaches in drug discovery and toxicology and introduced cell

culture methods, Integrated discrete Multiple Organ Co-culture (IdMOC) technology, stem cell applications, tissue engineering, organ-on-chip, human-on-chip technologies and high-throughput approaches to screening of potential drug candidates and risk assessment as well as non-mammalian model organisms such as *Caenorhabditis elegans*, zebrafish, drosophila and hydra. He explained how these alternatives could be equivalent to, or even better than, animal models. He quoted a passage from the Holy Quran stating that animals are like human beings and so need to be respected and treated with kindness. He stressed the need to work along the current trend of available and upcoming technologies in order to keep pace with global developments.

CME lectures

In the first lecture titled *Historical perspectives of alternatives to animal experiments*, Prof. **Mohamed Mobarak Hossein** traced the history of alternative methods, starting from the landmark book *The Principles of Humane Experimental Technique* by Russell and Burch in 1959. He explained that alternative methods have matured from a perceived or fabricated threat to biomedical research to an obvious opportunity for advancement without causing pain and distress to animals. Then, Dr **Mohamed T. Salman** spoke on *The Scope of Alternatives to Animal Experimentation in Pharmacology*. He described the way *in vitro* cell and tissue culture methods have reduced the use of rodents in the initial screening of potential new drugs. He also focused on examples of human skin equivalent tests (like EpiDerm, EpiSkin, SkinEthic, etc.) being used as alternatives to animal-based corrosive and irritation studies and of corneas from slaughtered cows or chicken eyes used for eye irritation studies. The final lecture was delivered on *The Conception of Animal Simulators from Idea to Product: A Challenging Journey* by a spokesperson of Elsevier who presented the way alternatives software is conceptualized and developed.

Hands-on training in alternatives software

The participants were divided into four groups. Parallel one-hour sessions were conducted at four different stations: The participants received hands-on training on simulators/software made in India, such as ExPharm, ExPhysio and ExCology, by the software originators, Prof. Ramaswamy Raveendran (Jawaharlal Institute of Postgraduate Medical Education and Research [JIPMER], Pondicherry), Prof. Sandhya T. Avadhany (St. John's Medical College, Bangalore), Prof. Mohammad A. Akbarsha (MGDC), Prof. S. K. Bajaj (Maulana Azad Medical College, New Delhi) and Prof. Chandragouda R. Patil (R. C. Patel College of Pharmacy, Dhule), in addition to other experts working in the same field.

Valedictory function

Prof. Mohammad A. Akbarsha, the Chief Guest, said he hoped that the workshop/CME had motivated the participants to employ alternatives to animal experimentation and stated that it is high time to adopt the available technologies, and also develop further technologies at affordable costs. Prof. Sandhya T. Avadhany and Prof. Ramaswamy Raveendran, Guests of Honor, congratulated the organizers on the well-organized workshop and appreciated the opportunity to learn about the e-resources. Dr Syed Z. Rahman, Organizing Secretary, thanked all the contributors, participants, administration and funding agencies for supporting and making this workshop successful.

Conclusion

At the end of the workshop, feedback was collected from participants. Most of them rated the workshop/CME at point 4 on the Lickert scale. The workshop was successful in creating awareness and enhancing acceptability of alternatives. The participants were willing to use modern techniques in the form of alternatives, and this program provided them a good platform towards this end. Most of the participants urged to have similar workshops on a regular basis.

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