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Preamble
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Lectures held at the 99th Indian Science Congress Plenary Session:
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Alternatives to Animals in Education, Research, and Risk Assessment: An Overview with Special Reference to Indian Context

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Summary
Animal experimentation remains a matter of debate and controversy. On one side is the opinion that animal use in education, research, and testing raises concerns relating to ethical treatment of animals, the source from which animals are obtained, ill-designed experiments, relevance of inferences from the testing outcomes, safety issues, biodiversity issues, environmental concerns, etc. On the other side is the opinion that the benefits that have come from animal research would not have been realized if not for animal experiments. These concerns led to the 3Rs principles of Replacement, Reduction, and Refinement in the use of animals in experimentation, otherwise known as the concept of alternatives. Once a matter of imagination, today science has evolved such that drugs, cosmetics, and agrochemicals can be tested adopting scientifically validated non-animal methods. Today, the science of alternatives has gained recognition from both an ethical and a scientific point of view. The breakthroughs in ICT, cell culture techniques, in vitro toxicology, molecular tools, functional genomics, tissue engineering, systems biology, etc., have made it possible to take the concept of alternatives from a myth to reality. There is still a long way to go, however: just as science has no end, the search for alternatives has no end.

Keywords: alternatives, in vitro systems, in silico approaches, validation of alternatives

1 Introduction

The use of animals in science is a contentious issue, with opinions from scientists, teachers, animal protectionists, and the public either to support animal experimentation or to reject/minimize it (Pawlik, 1998; Giridharan, 2000; Greek and Greek, 2010; Greek, 2012). The main purposes of animal experiments are for: basic biological knowledge; fundamental medical research; the discovery and development of drugs, vaccines, and medical devices; toxicity testing of drugs, other chemical entities, and consumer products; and education and training (Taylor et al., 2008). These contexts of animal use can be broadly classified into (i) education and training, and (ii) research and testing. It is roughly estimated that among the several million animals sacrificed for the cause of science, in any country, 1-10% are used in education and the rest in research and testing. There has been a movement against the use of animals in endeavors in science on several grounds and, as a result, greater awareness has been generated. A concerted effort across the globe has resulted in a considerable reduction in the number of animals used and increased recognition that animals are sentient beings. It is cause to rejoice that India has made rapid strides in phasing out animal use in education and training, but the scenario with respect to research and testing has changed very little. This article is an attempt to sensitize the stakeholders to the need to promote the most humane science.

2 On the use of animals in education and training in basic sciences and the potential alternatives

In education and training, wild-collected as well as laboratory-bred animals in millions are used in the name of knowledge acquisition and/or skill development in basic life sciences, as well as in professional education for medical, veterinary, pharmacology, etc., purposes. Dissections of and experiments using animals have been practiced as laboratory exercises in zoology and allied life sciences since the 1920s, at a time when the subjects of animal anatomy, taxonomy, and evolution were emphasized in theory courses. These classical subjects have now been relegated to the background in the light of emerging subjects such as biochemistry, molecular biology, and biotechnology, warranting lectures and laboratory exercises in these subjects. Further, the demand for animals for purpose of dissections has increased to such an extent that animals are no longer available in large enough numbers to meet the demand. The large-scale removal of animals from their natural habitats for an unnatural purpose can potentially lead to biodiversity loss and an ecological crisis. Laws have been enacted protecting selected animal species, but these laws are often not heeded, and animals have been continued to be used in education. Social groups and scientists also have questioned both the logic and the relevance of animal dissections in education in the contemporary
specialists from different countries met and set up a framework to Animal Testing (CAAT) organized a workshop in which quality learning (James, 2010). The Center for Alternatives surgery technology reality-based dissection simulator developed using virtual a more recent addition to this list, is an innovative virtual computer animation of the frog being cut. The student does only pre-recorded renderings and, again, every student sees the no physical simulation of tissue being cut or manipulated but in context, but the limitation here is that these products offer get the benefit of controlling the dissection and seeing content in three-dimensional space. The enhanced multimedia presentations include: Digital Frog by Digital Frog International; CyberEd Dissection Series by Plato; DissectionWorks by ScienceWorks; and Frogguts by Frogguts, Inc. These products allow a student to select a tool, such as a scalpel, and float the tool over a 2D representation of a frog. Clicking the mouse in the right place triggers a video clip or computer animation of the frog being cut. The student does get the benefit of controlling the dissection and seeing content in context, but the limitation here is that these products offer no physical simulation of tissue being cut or manipulated but only pre-recorded renderings and, again, every student sees the same sequence or set of images, texts, and videos. V-Frog™, a more recent addition to this list, is an innovative virtual reality-based dissection simulator developed using virtual surgery technology1, and it is highly efficient in achieving quality learning (James, 2010). The Center for Alternatives to Animal Testing (CAAT) organized a workshop in which specialists from different countries met and set up a framework for a comprehensive educational program, and a consensus was agreed upon for generating teaching materials pertaining to the 3Rs approach in education (Daneshian et al., 2011).

Given that many countries have dropped dissection of animals from the curriculum, the pace of change in India has been initially rather slow (Akbarsha, 2007; Venkatesha, 2007; Vasudevan and Supriya, 2011), although animal welfare groups and teachers from India have been demanding discontinuation of animal dissections in education. The Central Board of Secondary Education (CBSE), constituted by the Union Government of India, a Board of Education for public and private schools, stopped prescribing dissections in its curriculum, the first dramatic change in this connection in India. Gujarat, Haryana, Uttar Pradesh, West Bengal, Rajasthan, Chhattisgarh, Himachal Pradesh, and Tamil Nadu, to mention a few, also dropped dissections from the school curriculum of the respective State Boards. Mahatma Gandhi-Doerenkamp Center (MGDC) for Alternatives to Use of Animal in Life Science Education, located at Bharathidasan University, Tiruchirappalli2, which draws strength from a number of non-governmental organizations, is forging ahead with a movement in the country to curtail animal use in higher education (Akbarsha and Pereira, 2010; Akbarsha et al., 2010). The University Grants Commission (UGC) has been regularly reviewing the use of animals in life science education and, recently, brought up guidelines for discontinuation of dissection and animal experimentation in zoology/life sciences education in a phased manner3, a progressive effort in this direction. Implementation of these guidelines, and the change from “dead zoology” to “live zoology”, i.e., learning about the animals without removing them from their natural habitats, is imperative. Allowing the learner to visit the animals and make observations, will not only save the millions of animals being killed/sacrificed for purpose of higher education in India, but also will facilitate the introduction of ICT tools, the “alternatives”, in the pedagogy of laboratory exercises. Thus, India today can be proud of being the pioneer in formulating guidelines of this kind for other countries to adopt. Much depends on to what extent these guidelines will be adopted by the universities and colleges. This apprehension remains because there are still orthodox institutions, stubborn teachers, and old-fashioned academic bodies that are reluctant to change to the innovative pedagogy.

3 On the use of animals in education and training in biomedical sciences/pharmacology and the potential alternatives

Medical education in India previously required the use of animals such as frogs, rats, guinea pigs, dogs, etc., as tools for learning first-hand certain aspects of anatomy, physiology, biochemistry, pharmacology, surgery, etc. This use, from a global perspective, has emphasized two components – the acquisition of knowledge and the development of skills (Balcombe, 2000). It is a fact that this use has done well in developing skilled clinicians, surgeons, pharmacologists, and so on. However, those were the

1 http://www.tactustech.com/vfrog/
2 http://www.mgdcseaua.org/
3 http://www.ugc.ac.in/oldltpdf/commissiondecision/479.pdf
days when there were fewer institutions, fewer students, ethical concerns were rarely raised – or not raised at all – and no better learning/skill development modalities were available. With the increased number of institutions, increases in enrolment, and the recognition of animal sentence and the consequent ethical considerations, combined with the development of several “alternatives” through innovative technologies have resulted in a paradigm shift in the use of animals in medical education in Europe and the USA. The alternatives include the mannequins, simulators, virtual settings, human volunteers, self-experimentation, etc. Special mention should be made of the successes made using virtual reality simulators (VRS), human patient simulators (HPS), and minimally-invasive surgery (MIS) (Seymour et al., 2002; Good, 2003; Gallagher, 2005; Seymour, 2008). Studies have proved that learning and/or skill development adopting these various “alternatives” not only compensate for the real-time exposure to animal models, but the outcomes are much better (Dewhurst, 1994; Hughes, 2001). However, the pace of change in India has been rather slow (Roy and Tekur, 2001; Hariharan, 2004; Dhingra et al., 2006). The curricula still prescribe laboratory exercises using animals, and the training is mandatory, in spite of the recent liberal permission from the Medical Council of India (MCI) and the Pharmacy Council of India (PCI) that “alternatives” can replace animals in laboratory exercises. The conservatism in the Indian mindset has precluded, by and large, change to the modern and novel approaches in medical and pharmacy education. Concerted effort from MGDC, a few non-governmental organizations, and enlightened teachers should lead to a change to “alternatives,” and a desirable change is expected soon.

4 Use of animals in drug development and risk assessment

Animal experimentation remains a matter of debate and controversy. Those who are not in favor of animal use in drug discovery and risk assessment raise concerns relating to ethical treatment of animals, the source from which animals are obtained, ill-designed experiments, relevance/inferences of the testing outcomes, safety issues, etc. Those who are in favor animal testing point out the benefits that have come from animal research, such as organ transplants, open-heart surgery techniques, lifesaving drugs, effective insulin and cancer treatments, and animal and human vaccines. One cannot deny the contributions made by animals for the betterment of humans, but it should be realized that animal testing is not the gold-standard, and accurate prediction of drug toxicity on the human remains a major challenge in drug development (Li, 2004). Over the past decades, a number of drugs have been withdrawn due to adverse effects, sometimes fatal, on humans, despite having been declared safe after having passed all the animal tests required by the Regulatory Authorities. The cost of withdrawal of a drug from the market is astronomical, including losses in resources and time spent in drug development. It has been estimated at $800 million, with an average development time of 12-15 years required for a successful drug (Dimasi et al., 2003). Again, this cannot be considered as a gold standard (Morgan et al., 2011), as the development cost depends on a variety of factors (Dickson and Gagnon, 2004). A study reveals that out of 10,000 new chemical entities (NCE), only one may become a successful drug and, according to US Food & Drug Administration (FDA) statistics, only around one in every ten new medicinal products that progress to clinical trials ever reaches the registration stage\(^4\). Unfortunately, some of the drugs have caused horrific harm to people, even though they had all been “safety tested” on animals. Opren, hailed as a “wonder-drug” for arthritis, was withdrawn in 1982 after 62 deaths and 3,500 serious side effects in the UK alone, including damage to skin, eyes, circulation, liver, and kidneys\(^5\). Practolol, a selective beta blocker marketed under different names, was one of the biggest drug disasters, and it was withdrawn after reports of adverse drug reaction, including 40 deaths (Abraham and Davis, 2006). The famous case of thalidomide, administered to pregnant women as a harmless tranquilizer, caused innumerable birth defects worldwide. In December, 2004, Vioxx, the blockbuster drug from Merck & Co. Inc., was withdrawn from market due to serious cardiovascular events, including heart attacks and strokes (Mukherjee et al., 2001; Juni et al., 2004; Topol, 2004). But its withdrawal came too late, as 80 million patients had taken this medicine, with a 38,000 estimated dead (Juni et al., 2004). Had this not been reliant on animal testing, such disasters could have been avoided\(^6\). In fact, there is a long list of drugs that have been recalled – drugs that are withdrawn from the market and drugs for which safety alerts have been issued, either by US FDA or by other regulatory agencies\(^7\).\(^8\).\(^9\). (e.g., Wysowski and Swartz, 2005)

About 70 million chemicals have been synthesized, as registered in Chemical Abstracts Service. It is not really clear how many of these chemicals are found in consumer products, in the environment, or in our bodies. A reasonable estimate is that people are exposed to about 100,000 relevant synthetic chemicals, in contrast to the 5,000 to 10,000 for which actually (widely varying in depth) safety assessments exist. However, we are most likely exposed to an even larger number of chemicals, given all the naturally occurring sources (Hartung, 2010). The conventional method of assessing these chemical entities for toxicity, or subjecting them to safety and risk assessment, is to do animal tests. A variety of tests are conducted on a variety of animal species. Over and above the animals used in the preliminary screening, chronic toxicity testing, and risk assessment (hazard identification, hazard characterization, exposure assessment, and

\(^4\) http://www.fda.gov/ScienceResearch/SpecialTopics/CriticalPathInitiative/CriticalPathOpportunitiesReports/ucm077262.htm
\(^5\) http://www.dlr.org/resources/alternative.htm
\(^6\) http://www.pcrm.org/search/?cid=1172
\(^7\) http://www.fda.gov/safety/recalls/default.htm
\(^8\) http://www.fda.gov/dockets/dockets/98fr/100898b.txt
\(^9\) http://www.medindia.net/patients/patientinfo/drugs-banned-in-india.htm
risk characterization), the chemical is to be assessed for ADME, and then there is a battery of tests that need to be conducted for safety evaluation. These include carcinogenicity, teratogenicity, genotoxicity, reproductive toxicity, etc. (Blaauboer, 2002). The total animal requirement to subject all chemicals likely present in the environment, consumer products, and foods thus will be astronomical; to that extent, animals are subjected to pain, distress, and sacrifice. Added to this is the problem that the data generated in animal models do not fit well for the human context in view of species differences in metabolism of the drug/putative toxicant. The major issue here concerns the Phase I and Phase II metabolizing enzymes, more importantly Cyp450 isoenzymes, which differ between species (Bibi, 2008; Graham and Lake, 2008). An estimate made by Taylor et al. (2008) puts the global use of animals in experiments during 2005 alone at a little more than 115 million.

This only reiterates that it is important for scientists to make their choices with a scientific rationale, a sense of compassion and ethics regarding animal use, and then to look at the need/possibility of using better, more humane and more precise tools (Lord, 2004; NRC, 2007; Yang, 2009). Some of the pertinent questions in this connection are i) does research and testing really require this many animals; ii) can animal testing be conducted in a way that does not subject the animals to distress or pain; and iii) can testing be done without animals?

5 The 3Rs principles

It was precisely these three questions that led to the 3Rs principles of Replacement, Reduction, and Refinement in the use of animals in experimentation, otherwise known as the concept of “alternatives.” Science has evolved such that drugs, cosmetics, and agrochemicals now can be tested using scientifically validated non-animal methods, thereby saving/protection the lives of human beings and also saving the lives of several million animals from pain/distress and/or death. The alternatives have paved the way for better, more precise and more credible scientific investigations. In addition to the animal welfare aspects, alternatives offer advantages such as cost-saving and higher throughput.

The concept of the 3Rs was propounded in the book “The Principles of Humane Experimental Technique” by the British scientists W. M. S. Russell and R. Burch (1959). The word “alternative” was coined by D. H. Smyth (Smyth, 1978) to describe the 3Rs. Russell and Burch’s book seriously challenged the scientific community about the use of animals in research. The Declaration of Bologna in 1999 further reiterated the need and rationale for the scientific community to adopt alternatives\textsuperscript{10}.

Today, the science of alternatives has gained recognition from both ethical and scientific points of view. The breakthroughs in in vitro systems, in silico toxicology, molecular biological tools, omics approaches, tissue engineering, etc., have made it possible to take the concept of alternatives from the perspective of animal testing from myth to reality (Aardema and MacGregor, 2001; Kniewald et al., 2005; Bhogal et al., 2006; Kroeger, 2006; Chapin and Stedman, 2009; Hartung and Hoffmann, 2009; Elliott and Yuan, 2010; Roggen, 2011).

6 In vitro systems: alternatives to animal testing in risk assessment

Since alternatives to animal testing rely heavily on in vitro systems, albeit with some limitations (Roggen, 2011), it is pertinent to highlight some of the assays/discoveries that redefined the risk assessment process and, eventually, set the stage for more challenging research (Eisenbrand et al., 2002; Bernauer et al., 2005). Application of in vitro techniques has resulted in a sincere effort to find test methods capable of screening large numbers of chemicals so as to reduce animal testing and, at the same time, facilitate risk assessment strategies (Bal-Price, 2008; Coecke et al., 2007; Lein et al., 2007; Crofton et al., 2011). One of the most successful alternative tests has been the Limulus Amoebocyte Lysate assay (Pearson et al., 1985), which was developed as an alternative to the rabbit pyrogen test, saving one million rabbits annually. More recently, the human whole blood pyrogen test has proved to be a better means of finding pyrogenicity than the other tests (Hartung et al., 1998). Cell-based assays have been developed to evaluate the toxicity of compounds, and accurate, precise, and sensitive end-points have been introduced. Various cultures of brain cells have been used to deliver insights on CNS disorders and elucidate the pathogenesis of oxidative stress in neurodegenerative brain disorders, AIDS-associated brain pathology, drug abuse, and aging (Aksenova et al., 2005). Neuroblastoma or glioma cell lines can be used to investigate the interaction of neurotoxicants with ion channels or receptor and signal transduction systems, as well as with basic metabolic functions. Several neuroblastoma or glioma cell lines or PC12 cells have been used extensively in neurobiological studies, thus providing a large amount of information on their physiology and biochemical composition. Mouse neuroblastoma cell lines have been used in screening teratological compounds (Mummery et al., 1984) and in predicting acute toxicity of certain groups of substances (Walum and Peterson, 1983). Cultures of Schwann cells may represent a good model to study the effect of chemicals on myelination. However, according to Veronesi (1992), because of the complexity of neurons and the lack of a cell line to mimic the in vivo neuronal conditions, no single in vitro preparation can be relied upon to detect all possible end points. However, many studies are underway, and at least one study should prove promising. Scientists have developed a three-dimensional brain cell culture system that resembles brain tissue in many of its characteristics. This means that various aspects of ischemia research now can be investigated in the test tube instead of in animals. Due to its significance, this in vitro method was evaluated by the EU ACuteTox Project as a

\textsuperscript{10} http://www.icare-worldwide.org/indian_congress/bologna.html
In vitro genotoxicity assays based on the detection of mutagenicity in bacteria or chromosomal damage in mammalian cells have been useful in evaluating genotoxic, non-genotoxic, carcinogen or non-carcinogen compounds. The in vitro micronucleus assay is a mutagenicity test system used for the detection of chemicals that induce the formation of small membrane-bound DNA fragments such as micronuclei in the cytoplasm of interphase cells (Fenech, 2000). In recent years, scientists increasingly have seen the in vitro micronucleus test as an attractive tool for genotoxicity testing because of its simplicity and wide applicability in different cell types (Decordier and Kirsch-Volders, 2006). The assay is a potential method for genotoxicity/photogenotoxicity screening of drug candidates owing to reduced false positive results, and it requires a lesser amount of toxic compound (Witte et al., 2007). Moreover, the assay has been endorsed by ECVM and approved by REACH for regulatory use as part of the genotoxicity test battery (ECVM, 2006a), demonstrating the authenticity of the assay.

The study of carcinogenesis has been greatly facilitated by the discovery of in vitro morphological transformation of mammalian cells in culture. The most frequently used endpoint for cell transformation is morphological transformation, which involves phenotypic alteration in the cultured cells, such as alterations in cellular morphology, disorganized patterns of colony growth, and acquisition of anchorage-independent growth (Barrett et al., 1986). The most commonly used cell transformation assays are Syrian Hamster Embryo (SHE) assay, Balb/c assays, and C3H/10T1/2 assay. A study reveals the Balb/c assay as competent to evaluate the carcinogenic potential of chemicals and environmental mixtures. It is very likely that, on validation, it will replace animal testing in a two-stage cell transformation similar to two-stage carcinogenicity assays in vivo to detect both tumor promoters and weak initiators (Vanparys et al., 2010). The replacement of the classical acute toxicity test, or LD50, with the Acute toxic class method / Fixed dose procedure / Up-and-down procedure, as approved by OECD, can reduce animal use from 45 to 8 animals per test chemical, an illustrative example for reduction and refinement alternatives.

The Bovine Corneal Opacity and Permeability (BCOP) eye test has been validated by ECVAM and accepted as a screening test to detect ocular corrosives and severe irritants in a tiered testing strategy (ECVAM, 2007). The test substance is applied to isolated bovine cornea (obtained from slaughterhouses) and determination of irritation or corrosion is evaluated by measuring changes in opacity and permeability using an optical instrument. Isolated chicken eye (ICE) or chicken enucleated eye test (CEET) also has been approved by ECVM. All these tests are organotypic in vitro approaches to animal testing, and it has been estimated that there will be at least 10% reduction in the number of animals killed in the US alone.

Engineered skin/epithelial models have come to replace the animals in the skin sensitization/irritation/corrosion testing of cosmetics (Alder et al., 2010). A team of scientists working at L’Oréal, France, created a reconstructed skin model that has been validated by ECVM (ECVM, 1998) and approved as a replacement or refinement to animal testing for skin corrosivity. The product, marketed under the name of EPISKIN in Europe, is a huge success, both in terms of saving thousands of innocent animals and as a significant step forward in the field of science. The model is created on a base designed on human collagen on which adult keratinocytes are layered to get a 3D structure. The cells grow, divide, and then differentiate in the air-exposed culture. At the end, the epidermis consists of a mitotic layer, a mucous Malphigian layer and a functional horny layer. The test substance is either applied topically or in the culture medium, and the irritancy is identified by MTT assay. Studies have shown the reproducibility and reliability of EPISKIN in assessing toxicity of cosmetic and dermatological products (Roguet et al., 1994, 1998; Faller et al., 2002). Scientists are working to test the applicability of EPISKIN to examine whether reconstructed skin can be useful for prediction of the genotoxic effect of a compound (Flamand et al., 2006; Ouédraogo et al., 2007). The study was extended to discover whether this model can prove to be an effective barrier to toxic compounds. A specific co-culture system using target cells (lymphoma cells) cultured underneath the in vitro reconstructed skin (EPISKIN) was developed and a micronucleus assay was performed. The results concluded that EPISKIN is a metabolically competent tissue and a biological barrier mimicking realistic conditions of use (Flamand et al., 2006; Ouédraogo et al., 2007).

Similarly, another reconstructed skin model, called EpiDerm, has been validated (ECVM, 2000a). It closely parallels human skin, thus providing a useful in vitro means to assess dermal irritancy and toxicology (Cannon et al., 1994). It possesses a human 3D skin-like tissue structure with a complete stratified epithelium (Kandárová et al., 2007) and in vivo-like lipid profile (Ponec et al., 2002). Recent studies indicate that EpiDerm reproduces many of the barrier function properties of normal human skin. SkinEthic and CORROSITEX, the other models, developed on the same lines, have been validated and approved by the regulatory authorities (ECVM, 2006a,b). Skin sensitization is an immunologically-mediated cutaneous reaction to a substance. The classical Local Lymph Node Assay (LLNA) is an alternative method. The principle behind this test is that sensitizers induce a proliferation of lymphocytes in
the lymph node draining the site of chemical application (For more information on alternative methods, the reader may visit http://altweb.jhsph.edu).

7 In vitro-in vivo approach in drug testing and risk assessment

One of the major questions raised in connection with in vitro toxicology is that a cell does not represent the human body in its holistic perspective, i.e., cells of different kinds in tissues and organs, and therefore cell-cell interaction and multiple targets are not mimicked. Physiological situations where one organ doing the metabolic function and another organ responding to the metabolite(s) is not provided for in the conventional in vitro tests. It is known that species differences form the basis for the failure of several drugs. A large number of drugs and other chemicals have been shown to invoke the action of hepatic microsomal cytochrome P450 (CYP) isoforms in experimental studies (Bibi, 2008). Most CYP forms are induced by receptor-mediated mechanisms leading to an increase in gene transcription. Important nuclear receptors involved in the induction of CYP1A, CYP2B, CYP3A, and CYP4A subfamily forms comprise, respectively, the aryl hydrocarbon receptor, the constitutive androstane receptor, the pregnane X receptor, and the peroxisome proliferator-activated receptor alpha. Hepatic CYP form induction can be assessed by in vivo, ex vivo, and in vitro methods. Significant species differences can exist in the enzyme induction response to a given chemical and also in the toxicological consequences of induction. Hepatic CYP form induction in humans may lead to clinically important drug-drug interactions. In rodents, hepatic CYP form induction can be associated with the formation of tumors by non-genotoxic modes of action in the liver, thyroid, and other tissues (Graham and Lake, 2008).

A major challenge in drug development is to accurately estimate human adverse drug effects to allow the selection and advancement of drug candidates with the best safety profile for further development. Due to species differences, safety data obtained with the routine in vivo studies with nonhuman laboratory animals do not always correctly predict human outcome. Human liver-derived systems, especially human hepatocytes, represent physiologically relevant experimental systems for the evaluation of human adverse drug effects (Li, 2011). The assays developed with human-based in vitro experimental systems for the assessment of two major adverse drug effects – drug-drug interactions and drug toxicity – can be used routinely during drug development to select and optimize drug candidates to enhance the probability of clinical success. These issues are now addressed in cryopreservation of human primary hepatocytes and the Integrated Discrete Multiple Organ Co-Culture (Li, 2008). A novel three-dimensional (3D) tissue-engineered scaffold system in a miniaturized setting has been developed, which can mimic multiple organ interaction in an “organ-on-chip” set-up. This is expected to revolutionize in vitro toxicology (Hosseinkhani et al., 2008). Along similar lines, a well-defined synthetic peptide that can self-assemble into three-dimensional interweaving nanofiber scaffolds to form a hydrogel, PuraMatrix, as a substrate for hepatocyte culture has been developed. Freshly isolated primary rat hepatocytes attached, migrated, and formed spheroids within 3 days after seeding on PuraMatrix, providing for three-dimensional hepatocyte culture, which answered the question of whether the in vitro system provides for the same cell-cell interaction as in an organ in vivo in its three dimensional architecture (Wang et al., 2008).

8 Alternative model organisms

In vitro approaches have several advantages but come with some limitations too. The in vitro test will not and cannot mimic the in vivo test one hundred per cent. At the same time, dealing with mammalian models in vivo raises ethical and moral issues. Thus, the possibility of conducting the tests in vivo in simpler organisms that belong to the lower levels of the phylogenetic tree and whose genome scale data are available is a viable option. There was a time when non-mammals were thought to be far from ideal materials for the study of biomedical sciences. However, it has now become abundantly clear that some non-mammals are not only convenient materials but also are endowed with physiological and pharmacological properties common to humans (Peterson et al., 2008). Because genes, receptors, and molecular processes are highly conserved across animal phyla, studies with other animal species could be representative for “higher,” more complex animals (Hill et al., 2005). This approach is based on the level of sentence of the animal dealt with and also the capacity for self-renewal from ecological perspectives, combined with the availability in abundance, ease with which they can be raised and experimented upon, etc. To that effect, the zebra fish (Danio rerio), Drosophila (Drosophila melanogaster), the nematode worm Caenorhabditis elegans, and the simple cnidarian Hydra are now being proposed as in vivo alternative models that will be amenable to reduction and refinement alternatives.

The zebrafish, a small tropical fish native to the rivers of India and South Asia, has become one of the most popular model organisms in developmental genetics and (eco) toxicology. A number of unique features have contributed to its attraction, including its rapid development, easy maintenance in the laboratory, large number of offspring, transparency of embryos, and access to experimental manipulation. The genomic sequencing of zebrafish is almost complete and it is used as a model for human disease and development. Zebrafish embryos are now models for studying the effect of chemicals on gene and protein patterns, as well as the potential implications of differential expression for toxicity. These fish also are suitable for toxicokinetics, toxicodynamics, transcriptomics, toxicogenomics, proteomics, and metabolomics studies. The animal also offers biomarkers of endocrine disruption, immune modulation, genotoxicity, or chronic toxicity (Hill et al., 2005; Peterson et al., 2008; Scholz et al., 2008). Zebrafish can be
used to eliminate potentially unsafe compounds rapidly in the early stages of drug development and to prioritize compounds for further pre-clinical studies (McGrath and Li, 2008). The Vitellogenin1 mRNA induction assay has been proved to be a sensitive biomarker of exposure to organochlorine pesticides and brominated flame retardants (Chow et al., 2012). The zebrafish model has great potential for future applications in developmental toxicity testing in the context of REACH legislation, given that approximately 30,000 chemicals may need to be tested for safety, and under current guidelines such testing would require the use of approximately 7.2 million laboratory animals of which more than 80% would be used for examining reproductive and developmental toxicity (Lee et al., 2012). The zebrafish is a powerful whole animal model, complementary to in vitro and mammalian models in high-throughput behavioral screening of compound libraries (Ali et al., 2012). A recent report claims that zebrafish possess great promise in evaluating the toxicity of nanoparticles as well (Liu et al., 2012).

Drosophila has remained the most pioneering model organism for understanding concepts in genetics and developmental biology, including human diseases and toxicological research (Siddhique et al., 2005). Drosophila is the closest invertebrate model organism to humans, based on sequence similarity/conservation (reviewed in Tiwari et al., 2011). Its recently discovered application as a model organism in toxicology, especially in the context of NRC’s vision of toxicology for the 21st century, has earned a new status for this fly; a new branch of toxicology, “Drosophotoxicology,” has emerged (Rand, 2010). Its entire genome sequence has been analyzed, and its four chromosomes accommodate approximately 13,600 genes (Adams et al., 2000). Drosophila, with several distinctions in terms of biology and life cycle, provides great advantages for testing the variety of chemical toxicants to be investigated, the mode of delivery to the organism, the developmental stage, and the end points to be measured in determining biological/toxicological effects (Rand, 2010). Earlier, Drosophila served as an excellent in vivo model for genotoxic assessment (Siddhique et al., 2005). Drosophila fulfills most of the requirements of ECVAM, which recommended Drosophila as one of the promising organisms that will help answer complex questions in simple organisms (Tiwari et al., 2011).

Caenorhabditis elegans, the little nematode worm, has provided the discovery tools for several outstanding concepts in biology, particularly neuroscience, development, signal transduction, cell death, aging, and RNA interference, cell tracking, apoptosis, etc. (Antoshechkin Sternberg, 2007). The success of C. elegans as a model has attracted increased attention in the fields of biomedical and environmental toxicology. In fact, C. elegans has a number of features that make it not just relevant but quite powerful as a model for biological research. Its features have led to an increasing use of C. elegans in toxicology, both for mechanistic studies and for high-throughput screening approaches (Leung et al., 2008; Helmcke et al., 2010). In NRC’s recommendation on alternatives to higher animal models, C. elegans occupies a promising place. It is an attractive model because of its well-characterized and evolutionarily-conserved biology, low cost, and ability to be used in high-throughput screening (Boyd et al., 2010a, b). C. elegans is amenable to genetic manipulations such that it can be useful in unraveling mysteries in toxicology. For example, C. elegans expressing firefly luciferase has been developed, and it is useful in assessing effects of sub-lethal chronic exposure to environmental pollutants. Whole animal bio-luminescence is a valid toxicological endpoint and a rapid and sensitive predictor of toxic effects (Lagido et al., 2009). The advantages, such as conservation of disease and stress response pathways, availability of mutant and transgenic strains, and the wealth of biological information have led to increased use of C. elegans in toxicological studies (Boyd et al., 2012). Also, C. elegans contains cellular detoxification systems, including glutathione, metallothioneins, pumps and transporters, and heat shock proteins to regulate intracellular metal levels, and so provides several advantages for deciphering the mechanisms of metal detoxification (Martinez-Finley and Aschner, 2011). Most importantly, the toxic responses in C. elegans and mammalian toxicity were compared, and it was found that many endpoints were similar, indicating that measurements of morbidity and mortality in conjunction with morphology analysis in C. elegans may have the potential to predict mammalian toxic responses (Hunt et al., 2012). Thus, C. elegans represents an excellent complement to in vitro or cell culture-based systems and in vivo vertebrate models (Leung et al., 2008).

Hydra is a very simple eumetazoan diploblastic organism belonging to the phylum Cnidaria. Inhabiting freshwater bodies, this microscopic organism is by habit sedentary, attached to stones, pebbles, and plants. The cylindrical body has a basal disc with which it attaches to the substratum; at the opposite end is the mouth, located on top of a conical manubrium surrounded by 5-8 tentacles. The body has outer ectoderm/epidermis and inner endoderm/gastrodermis. Both layers are formed of musculo-epithelial cells but differ in finer details of structure as well as function. The primary method of reproduction is by budding, but it can reproduce sexually through development of transient gonads and the execution of fertilization between sperm and ova followed by the hatching out of a planula larva that undergoes encystment to overcome unfavorable conditions. Hydra has enormous powers of regeneration, and thus it has been a model organism for understanding regeneration, polarity, pattern formation, etc. This unique property is provided by the stem cells in Hydra, and the stem cell-nest renders Hydra an immortal animal. These aspects of simplicity have attracted the attention of biologists, and Hydra has since been a model organism in toxicity testing as well. Hydra spp has recently gained increased attention in aquatic toxicology as a sensitive and possible target species (Pascoe et al., 2003; Segner et al., 2003). Xenobiotic biotransformation, oxidative stress, growth, asexual reproduction, morphological changes, and feeding behavior could be conveniently determined in this organism. These endpoints represent key targets that underlie survival, growth, and reproduction, which form the basis of environmental risk assessment strategies (Quinn et al., 2012). In as much as morphology, regeneration, reproduction, feeding,
and attachment could be endpoints of convenience, biochemistry and bio-transformation of xenobiotics also are suitable targets to rely upon. Hydra is sensitive to metals, many organic toxicants, endocrine disrupting compounds, pharmaceuticals, nanomaterials, and industrial and municipal effluents. Drug/toxicant metabolizing enzymes have been identified in Hydra and are molecularly characterized (Dash et al., 2006, 2007). Above all, the toxicity endpoints now can be traced to the genes, since its full genome has been deciphered (Chapman et al., 2010). Hydra is so much a standardized lower animal model in aquatic toxicity testing that it was used in the WaterTox network, an international network of laboratories from eight participating countries that examined the applicability of a battery of simple, inexpensive bioassays in environmental management and the relevance of the test results in establishing the toxicological quality of water sources and drinking water (Dutka, 1989; Trottier et al., 1997). Hydra has become so popular in the arena of toxicological testing that it has staked a claim for appropriately designed, relatively simple, and inexpensive laboratory toxicity tests using it with a selection of acute and sub-lethal endpoints that are generally adequate, with small application factors, for predicting the environmental risk of polluting chemicals to freshwater ecosystems (Quinn et al., 2012).

9 In silico approaches

In silico toxicity is an approach to making precise and robust toxic predictions that can improve the risk assessment process while reducing the time span and economic burden accrued due to animal and/or in vitro testing. Hartung and Hoffman categorized in silico toxicity tools into 9 groups and identified QSARs as “the most prominent in silico tools at the moment” (Hartung and Hoffman, 2009). Quantitative Structure Activity Relationships (QSARs) is a mathematical modeling tool that has come into application to predict biological (toxic/pharmacological) activity associated with chemical structure. A set of parameters called descriptors is required to predict the toxic outcomes, such as genotoxicity, aquatic toxicity, and reproductive toxicity, to name a few. Different QSARs are employed depending on what type of toxic testing prediction is warranted, ebTrack, a new in silico tool, was developed as an integrated bioinformatics system for environmental research and analysis by addressing the issues of integration, curation, management, first level analysis, and interpretation of environmental and toxicological data from diverse sources. It is based on enhancements to the US FDA-developed ArrayTrack™ system through additional analysis modules for gene expression data, as well as through incorporation and linkages to modules for analysis of proteomic and metabolomic datasets that include tandem mass spectra (Chen et al., 2009).

ToxCast is another bio-informatics approach that was launched in 2007 by the US EPA and it is useful in developing ways to predict potential toxicity and to develop a cost-effective approach for prioritizing the thousands of chemicals that need toxicity testing. ToxCast helps explain how human body processes are affected by exposures to chemicals, and it helps determine which exposures are most likely to lead to adverse health effects. ToxCast testing methods include more than 650 high-throughput assays that can screen 2,000 environmental chemicals for potential toxicity. Phase I, Proof of Concept, was completed in 2009. It profiled more than 300 well-studied chemicals (primarily pesticides). These chemicals have more than 30 years’ worth of existing toxicity data, since they have been tested already using traditional toxicology methods (primarily animal studies). Data from animal studies can be searched and queried using EPA’s Toxicity Reference Database (ToxRefDB). Phase II currently is screening 1,000 chemicals from industrial and consumer products, food additives, and drugs.

The discovery of E-cell is expected to revolutionize the prediction of toxicity. The E-cell is a simulation package in which scientists modeled a hypothetical cell to carry out in silico experiments relating to protein-protein interactions, protein-DNA interactions, regulation of gene expression, and other features of cellular metabolism (Tomita et al., 1999; Takahashi et al., 2003). In as much as the potential of E-cell is being realized, further studies are being carried out to prove its applicability in the toxicological domain.

10 The EU Directives and legislation

Europe and the USA are forerunners and strong proponents of the alternatives concept. The EU Directive 86/609/EEC on “the protection of animals used for experimental and other scientific purposes” was amended and replaced by the Directive 2010/63/EU, which requires in Article 4: “Member States shall ensure that, wherever possible, a scientifically satisfactory method or testing strategy, not entailing the use of live animals, shall be used instead of a procedure”; and in Article 47: “The Commission and the Member States shall contribute to the development and validation of alternative approaches which could provide the same or higher levels of information as those obtained in procedures using animals, but which do not involve the use of animals or use fewer animals or which entail less painful procedures, and they shall take such other steps as they consider appropriate to encourage research in this field.”

In the realm of cosmetics, the Seventh Amendment to the EU Directive 76/768/EEC (the Cosmetics Directive) has become one of the most appreciated directions in favor of alternatives. It states that, as of 11th March 2009, Member States shall prohibit the marketing of cosmetic products containing ingredients or combinations of ingredients that have been the subject of animal testing using a method other than an alternative method. Three animal tests are exempt from this ban: repeated-dose toxicity, reproductive toxicity, and toxicokinetics, for which alternatives are not currently available and which can be used until a final cut-off date of March 2013, at which point they also will be banned.

http://www.epa.gov/ncct/toxcast/
11 Agencies that validate alternatives

The European Centre for the Validation of Alternative Methods (ECVAM) was established in 1992 with the mandate to coordinate and promote the development and use of alternatives in basic and applied research and regulatory testing. It also helps promote dialogue among legislators, regulators, and all relevant stakeholders, particularly industry, biomedical scientists, consumer organizations, and animal-welfare groups, with a view to the development, validation, regulatory acceptance, international recognition, and application of alternative approaches. ECVAM has already validated fifty-five alternative methods that have reached the market, and more yet are in the pipeline.

Similarly, the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) was established in the USA in 1997 under the NTP Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) as an interagency committee of the US Government. ICCVAM coordinates interagency technical reviews of new and revised safety testing methods with regulatory applicability, including alternative test methods that may reduce, refine, or replace the use of animals in order to advance animal welfare while ensuring human health and safety. The Japanese Center for Validation of Alternative Methods (JaCVAM) was established in Japan with a mission to promote the 3Rs in animal experiments for the evaluation of chemical substance safety in Japan and to establish guidelines for new alternative experimental methods through international collaboration. The Republic of Korea's Centre for the Validation of Alternative Methods (KoCVAM) is a recent addition to this list. International Cooperation on Alternative Test Methods (ICATM) was officially created in April 2009, when an agreement was signed between validation bodies from Europe, USA, Canada, Japan, and Korea. A Brazilian Center for Validation of Alternative Methods (BraCVAM) is underway.

In light of the EU Directives, many companies are proactively choosing to eliminate animal and human clinical testing due to ethical considerations. Here, alternative methods are urgently needed to replace existing animal tests. It is very encouraging that the development of many promising in vitro methods to evaluate skin sensitization is moving forward steadily. ECVAM has initiated formal pre-validation of the human cell line activation test (h-CLAT), the myeloid U937 skin sensitization test (MUSST), and the direct peptide reactivity assay (DPRA). Each of these assays has entered the pipeline. KeratinoSense assay, a cell-based reporter gene assay, has forged ahead and soon will be subjected to ECVAM review. IVIVS, a non-governmental organization based in Gaithersburg, MD, USA, is playing an important role in this endeavor.

The Centre for Documentation and Evaluation of Alternatives to Animal Experiments (ZEBET), established in Germany in 1989, aims to bring about the replacement, particularly of legally prescribed animal experiments, with alternative test methods, to reduce the number of test animals to the absolutely necessary level, and to alleviate the pain and suffering of animals used in experiments.

12 REACH and its contribution to risk assessment

REACH (Registration, Evaluation, Authorization and Restriction of Chemicals), EU, and ECHA (European Chemicals Agency) under it, aim to improve the protection of both human health and the environment while maintaining the competitiveness and enhancing the innovative capability of the EU chemicals industry. REACH makes industry responsible for assessing and managing the risks posed by chemicals and providing appropriate safety information to their users. According to REACH, as many as 30,000 single chemicals are required to register with a potential risk assessment requirement based on substance-specific data for the persistence, bioaccumulation, and toxic (PBT) properties for several thousand chemicals. There are two major issues here: there are more than this number of single chemicals that would potentially affect human health, and given the number of tests to be conducted and the volume of data that needs to be generated, the target cannot be achieved anywhere near the deadline date. The situation becomes ever more complex, since new chemicals will arise by the time evaluation of the existing chemicals is completed (Hartung and Rovida, 2009). From the perspective of “alternatives,” this has introduced a challenge for industry, the regulatory agencies, and the toxicological research community to develop and validate alternative high-throughput testing strategies and limit the testing with animals by implementing the reduction, refinement, and replacement (the 3Rs) principle in ecotoxicological testing. In order to provide for safety evaluation data for this many chemicals – and perhaps more – the search is on for non-animal (alternative) testing methods for REACH (Lilienblum et al., 2008).

13 Non-governmental organizations and initiatives

In the field of basic biomedical research, the Center for Alternatives to Animal Testing (CAAT), established at Johns Hopkins University Bloomberg School of Public Health, U.S.A., aims to promote humane science by supporting the creation, development, validation, and use of alternatives to animals in research, product safety testing, and education.
CAAT seeks to effect change by working with scientists in industry, government, and academia to find new ways to replace animals with non-animal methods, reduce the numbers of animals necessary, or refine methods to make them less painful or stressful to the animals involved\textsuperscript{20}. The Center for Alternatives to Animal Testing, Europe (CAAT-EU) was founded on a collaboration between the Johns Hopkins Bloomberg School of Public Health and the University of Konstanz. CAAT-EU will coordinate transatlantic activities to promote humane science in research and education, and will participate, as partner or coordinator, in publicly and privately funded European projects\textsuperscript{21}.

Several European non-governmental programs are also helping to steer the development of new approaches to toxicology. AXLR8, an EU project, seeks to accelerate the transition to a toxicity pathway based paradigm for chemical safety assessment by providing a forum for networking, information exchange, and collaboration\textsuperscript{22}. The Risk Assessment in the 21\textsuperscript{st} Century Project (Risk21), sponsored by the ILSI Health & Environmental Sciences Institute, part of the International Life Sciences Institute, is trying to look at the whole picture. In Risk21, approximately 90 scientists from industry, academia, government, and non-governmental organizations participate in four project teams that are focusing on ways to characterize real-world chemical exposure, incorporate dose-response information into risk assessment, develop a framework for toxicity testing, and account for the cumulative risk of exposure to multiple agents\textsuperscript{23}.

U.K.-based Unilever’s skin sensitization program is developing \textit{in vitro} and computational methods to assess the risk of contact dermatitis. To do this, the Unilever group is working to understand the biology of allergic responses in human skin and incorporating what they learn into computational models of the process\textsuperscript{24}.

The role that metabolomics has in the evaluation of xenobiotic toxicity studies is presented here, giving new ground to toxicology research. To provide a comprehensive assessment of this approach, the Consortium for Metabonomic Toxicology (COMET) has been formed by six pharmaceutical companies and the Imperial College of Science, Technology and Medicine (IC), London, U.K.\textsuperscript{25}. The objective of this group is to define methodologies and to apply metabolomic data generated using NMR spectroscopy of urine and blood serum for preclinical toxicological screening of candidate drugs. This is being achieved by generating databases of results for a wide range of model toxins that serve as the raw material for computer-based expert systems for toxicity prediction. With the completion of 147 studies, the chief deliverables of a curated database of rodent biofluid NMR spectra and computer-based expert systems for the prediction of kidney or liver toxicity in rat and mouse based on the spectral data have been generated and delivered to the sponsoring companies. The project has met and exceeded all of its targets, and it was judged a resounding success by the sponsoring companies who are, in many cases, already enhancing and making use of the data in their in-house studies (Lindon et al., 2005).

Just as scientists raced to define the human genome, the Human Toxome Project (HTP) at Environmental Working Group is working to define the human toxome – the full scope of industrial pollution in humanity. HTP scientists use cutting edge biomonitoring techniques to test for industrial chemicals such as bisphenol A and perchlorate that enter the body through pollution or even as ingredients in everyday consumer products\textsuperscript{26}.

The Sens-it-iv project, aimed at novel testing strategies for \textit{in vitro} assessment of allergens, has been launched to develop \textit{in vitro} alternatives to animal tests currently used for the risk assessment of potential skin or lung sensitizers. The project participants include 28 groups overall, of which 9 represent industry, 15 represent universities or research institutes, and 4 represent organizations\textsuperscript{27}.

14 The NRC and toxicity testing in the 21\textsuperscript{st} Century

Advances in molecular biology and toxicology are paving the way for major improvements in the evaluation of the hazards posed by the large number of chemicals found at low levels in the environment. The National Research Council was asked by the U.S. Environmental Protection Agency (US EPA) to review the state of the science and create a far-reaching vision for the future of toxicity testing. There was a landmark publication by the National Academy of Science (NAS) in June 2007, under the auspices of the National Research Council (NRC), \textit{Toxicity Testing and Assessment in the Twenty-first Century: A Vision and a Strategy} (NRC, 2007). This report advocates sweeping changes in regulatory toxicity testing. It envisages a shift from current whole-animal based systems to testing founded primarily on \textit{in vitro} methods, human cells in culture, \textit{in silico} biokinetic modeling, and mechanisms of toxicity as understood through systems biology. The EPA, the National Toxicity Program (NTP), and the National Institutes of Health (NIH) signed a memorandum of understanding to develop and implement new high-throughput \textit{in vitro} methods for testing chemicals and drugs. This will allow the collection of data requested in the NAS report. This significant step toward US development of alternative methodologies reflects both the best science and the most humane science.

\textsuperscript{20} http://caat.jhsph.edu/
\textsuperscript{21} http://cmc.uni-konstanz.de/leist/caat-europe/
\textsuperscript{22} http://www.axl8r.com/
\textsuperscript{23} http://www.risk21.com/
\textsuperscript{24} http://www.unilever.com/sustainable-living/
\textsuperscript{25} http://bc-comet.sk.med.ic.ac.uk/
\textsuperscript{26} http://www.ewg.org/sites/humantoxome/
\textsuperscript{27} http://www.sens-it-iv.eu/
To help implement the recommendations of the NAS report as quickly as possible, the Doerenkamp-Zbinden Foundation (DZF) and CAAT collaborated to establish the Transatlantic Think Tank of Toxicology, or T³. The T³ is an effort, shared among CAAT at Johns Hopkins University, the University of Konstanz in Germany, and Utrecht University in the Netherlands, which seeks to advance evidence-based toxicology.

The Human Toxicology Project Consortium (HTPC) is a group of stakeholders currently drawn from the corporate and public interest communities who share the objective of accelerating implementation of Toxicity Testing in the 21st Century. The Consortium believes that the NRC vision is best implemented through a large-scale, international, coordinated effort similar to the Human Genome Project of the 1990s. This effort is called the Human Toxicology Project. The mission of HTPC is to serve as a catalyst for the prompt, global, and coordinated implementation of “21st century toxicology,” which will safeguard human health and hasten the replacement of animal use in toxicology. Its vision is a global paradigm shift to an in vitro approach to the risk assessment of chemicals and drugs that is based on a modern understanding of human biology and disease pathways, yielding results more rapidly and more predictive of human health effects than current approaches. It includes Dow Chemical, DuPont, Exxon Mobil, The Hamner Institutes for Health Sciences, the HSUS family – Humane Society of the United States, Humane Society International, and Humane Society Legislative Fund, Johnson & Johnson, L’Oréal, Procter & Gamble, and Unilever as the partners. CAAT is an external partner. The key aims include technical (help promote the establishment and implementation of an international research roadmap), public policy (promote the development, adoption, and implementation of the new methodology through legislative appropriations and regulatory advocacy on a global basis), communication (promote greater appreciation of the need for a prompt and global transformation to the new paradigm among diverse stakeholders), and collaboration (build strategic partnerships to advance the vision of the Consortium, and, where appropriate, expand the Consortium).

15 World and national congresses

Eight World Congresses on alternatives have been conducted, the latest being the “Eighth World Congress on Alternatives and Animal Use in the Life Sciences” organized at Montreal, Canada, on Aug 21-25, 2011. National Congresses have been conducted in several countries, including India. These achievements were made possible by the aggressive campaigning of and benevolent contribution from philanthropic organizations such as Fund for the Replacement of Animals in Medical Experiments (FRAME), UK, Doerenkamp-Zbinden Foundation (DZF), Switzerland, 3R Foundation, Switzerland, Humane Society of the United States (HSUS), American Anti-Vivisection Society (AAVS), International Centre for Alternatives in Research and Education (I-CARE), People for Ethical Treatment of Animals (PETA), etc., which fund hundreds of projects pertaining to the innovation of new testing methods based on the 3Rs principle.

16 Scientific journals on alternatives

Scientific journals specifically dealing with animal alternatives include Alternatives to Animal Testing and Experimentation (AATEX), Japanese Society of Alternatives to Animal Experimentation (JSAAE), Alternatives to Laboratory Animals (ATLA), Alternatives to Animal Experimentation (ALTEX), In Vitro Cellular and Developmental Biology – Animal, and Toxicology In Vitro.

17 Conclusion

The 3Rs have steadily become more widely known and better appreciated, although much still has to be done to ensure their broader implementation. The discussion of issues such as unnecessary use or reasonable alternatives and their interpretation according to the law is essential for a critical, workable, and consensual approach to the 3Rs principle. At the same time, pertinent and well-designed alternative experiments, political support, financial assistance and technical acumen are to be brought in for the development and advancement of the alternatives dogma. More important is for those countries that are lagging behind to catch up and realize that “alternatives” is not just humane science but better science. It is also time for India to catch up with the international trend and for the regulatory authorities to approve adoption of the validated alternative methods in drug testing and toxicology.
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From Alternative Methods to a New Regulatory Toxicology

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Summary

The 3Rs concept to replace, reduce, and refine animal experiments celebrated its 50th anniversary in 2009. Meanwhile, a mechanistic toxicology has evolved that effectively relies to a large extent on methodologies that substitute or complement traditional animal tests. The biotechnology and informatics revolution of the last decades has made such technologies broadly available and useful. Regulatory toxicology has only slowly begun to embrace these new approaches. Major validation efforts, however, have delivered the evidence that new approaches do not lower safety standards and can be integrated into regulatory safety assessments. Political pressures such as the REACH legislation and the 7th amendment to the cosmetics legislation further prompt the need for new approaches, especially in the EU. In the US, the NAS vision report for a toxicology in the 21st century and its most recent adaptation by the EPA have initiated a debate regarding their toxicity testing strategy and how to create a novel approach based on human cell cultures, lower species, high-throughput testing, and modeling. The human toxome, a systematic mapping of the entirety of pathways of toxicity, is now underway. The lessons learned from the development, validation, and acceptance of alternative methods for the creation of a new approach to regulatory toxicology are reviewed herein. Besides the technical development of new approaches, a case is made that we need both conceptual steering and an objective assessment of current practices by evidence-based toxicology. The application of an approach modeled on Evidence-based Medicine (EBM) has been suggested, as for the last two decades EBM has demonstrated that rigorous systematic reviews of current practices and meta-analyses of studies offer powerful tools to provide health care professionals and patients with the current best scientific evidence for diagnostic and treatment options. Similarly, a portal for high-quality reviews of toxicological approaches and tools for the quantitative meta-analyses of data promise to serve as a door opener for the new regulatory toxicology. The Evidence-based Toxicology Collaboration was created in the US in 2011, and a European equivalent in 2012.

Keywords: evidence-based toxicology, regulatory toxicology, human toxome

1 Introduction

In the last 150 years, chemists have synthesized about 70 million substances. More than 100,000 of these are found in consumer products of daily use, in drugs, in cosmetics, in detergents, in our food, our clothes and – last but not least – as contaminants of our natural environment. When Bayer brought Aspirin™ – our eldest synthetic drug – to the market 111 years ago, no regulatory safety assessments on animals were stipulated by legislation. The producer was held liable for any problems with his products. This did not always turn out as positive as in case of aspirin; only a few days after aspirin, the same chemist, Felix Hoffmann (1868-1946), synthesized a sedative for coughs: heroin… (Fig.1). Not every product is as harmless as aspirin. It is easy to understand that, with each safety scandal, the desire for safety assessments grows. In the 1920s, scientists started to use mice and rats broadly for laboratory research. Until then, it was considered absurd that these animals could mirror humans. It was clearly convincing, however, just how fast experiments could be performed with them: the animals did not cost a lot, they reproduced quickly, and a large number of them could be kept in a small cage. This created a real research rush, similar to today’s introduction of stem cells.

With every scandal the toolbox of toxicology grew as chemists sought to prevent a similar occurrence. In the early 1930s in the US LashLure created a scandal: The cosmetic product was used to dye lashes permanently; unfortunately, the anilin dye it contained sometimes led to strong inflammation. More than 3,000 reports of collateral effects were collected: five women were blinded, and one woman died. This prompted the first regulation of cosmetics, which have since been controlled in the US by the FDA (Food and Drug Administration). Their employee, John H. Draize (1900-1992) in 1944 developed the Draize rabbit eye test, where a chemical is applied into the eye of a rabbit (Hartung et al., 2010). Today, many perceive this procedure as cruel, but in fact, for 65 years this test prevented the recurrence of a case like LashLure. In this manner, toxicology grew with every scandal, pieced together like a patchwork quilt.
The thalidomide (Contergan™) scandal of the 1960s (Fig. 2) led toxicologists to test for malformation of embryos, for example.

2 The birth of doubt in animal experiments

Concerns about animal experimentation and the killing of animals have a long history. Even the ancient Greeks discussed whether we should kill animals. In Germany in the 1920s there were 700 animal welfare associations. However, it wasn’t until 1959 that Bill Russell and Rex Burch in England developed what they called “the principles of humane experimental technique” (Russell and Burch, 1959). They referred to these principles as “the 3Rs,” i.e., Replacement, Reduction, and Refinement. One must substitute animals with non-sentient test systems (Replacement), when an alternative exists. One must reduce the number of animals used wherever possible, if the same result can be obtained with fewer animals (Reduction). One must avoid unnecessary suffering and distress by using, for example, analgesics or working under narcosis (Refinement). Any suggestion of Replacement was considered utopian 50 years ago. At that time, cell culture and computer programs were in their infancy, and few scientists could imagine that such methods might lead to success. Over the last few decades, however, industry, science, and politics have demonstrated a commitment to the 3Rs that has led to compromise with those who would prefer to see animal experiments end today rather than tomorrow. This represented the basis for a credible investment in overcoming animal testing. In fact, animal experiments decreased until the turn of the century by an estimated two-thirds since their peak in the mid-Seventies. Since then, however, numbers have been increasing again, due largely to the new techniques for manipulating individual genes in mice, which have become very popular scientific models.

In the meantime, we have a number of examples that 3Rs approaches have indeed come to fruition. For example, the LD₅₀ test has been used since the 1920s. This test determines the lethal dose of a chemical that kills 50% of treated rats. Until 1989, 150 animals per substance were used for this purpose (10 female and 10 male at 7 dosages each, plus one untreated control group of 10 animals). This resulted in an enormous number of animals being used, especially since almost any substance going to the market was tested. Apparently, the lethality of this test led to labeling with the famous skull and crossbones as an indicator of poison. Both the protection of workers and safety measures for the transport of substances also were determined on this basis. In 1989, after an analysis of test data, a revision of guidance took place on the OECD (Organization for Economic Development).
Co-operation and Development) level. The OECD, now 34 industrialized countries, achieved agreement to drastically reduce animal numbers. Since then, groups of 5 animals of one gender have been used, thus reducing the number of rats from 150 to 45 per substance. In the 1990s, a further step was taken. The idea was simple: Why should all animals be treated simultaneously? When starting with just one dose, a higher dose can be tested next if animals survive. If the animals die, the dose has to be lowered. At the same time, it was shown that groups of three rats suffice. Consequently, three methods were accepted internationally in 2001. On average, these tests use only 8 to 12 animals. From 150 to 45 to just 8 to 12 animals – an enormous reduction indeed. In addition, one of these methods introduced the notion that the animal does not have to await death but rather can be euthanized humanely when it shows signs it will not survive or will be severely damaged. This is an example of “Refinement” – the second R for the amelioration of pain and distress in animal experiments. Another classical example is testing for skin allergy. Traditionally, this has been done with guinea pigs. The Local Lymph Node Assay (LLNA) represents both a reduction and a refinement alternative in mice, as it uses fewer animals, involves a shorter treatment period, and ends the experiment at the stage of lymph node swelling instead of waiting for the skin lesions to occur.

3 We can do it differently – the replacement of animal experiments

Increasingly, animal tests in toxicology can be fully substituted – the third R for “Replacement.” As an example, human skin obtained from surgical procedures can be grown further in the laboratory. A small tissue sample can produce several square meters of skin. These technologies were developed originally for skin transplantation after burn injury, for example. Quickly, the idea arose that this tissue also could be used for testing chemicals. In fact, it was possible to demonstrate that artificial human skin is as suitable as rabbit skin to test skin corrosion or irritation by chemicals. The respective international test guidelines have been agreed upon. This was not only a milestone for the cosmetic industry (Hartung, 2008b), but also a proof-of-principle that international consensus can be achieved regarding the replacement of an animal test with an animal-free method (Hartung and Daston, 2009).

4 Validation of alternative methods – animal welfare must not impair safety

The prerequisite for the acceptance of 3Rs approaches, however, is that these approaches must not lower safety standards for consumers. For this reason, the concept of formal validation was introduced. In 1991, the European Center for the Validation of Alternative Methods (ECVAM) was created for this purpose in Ispra, Italy. About 50 alternative methods have been validated there so far, and a number are currently undergoing ring trials and peer-reviews. An American (1995), a Japanese (2005), and a Korean (2011) and Brazilian (2011) equivalent followed, and the creation of similar centers currently are being discussed in India and China.

When validating an alternative method today, several things have to be shown (Bottini and Hartung, 2009): (1) Has the method been clearly defined – especially, is it clear when to use it and when not? (2) Does the method have a scientific basis that reflects our understanding of pathophysiology in humans and in animals? (3) Is the method reproducible, i.e., do we get the same results when repeating the method in other laboratories? (4) Are the results of the method relevant, i.e., in general: Can the method predict the outcome of the traditional (animal) test (Hartung et al., 2004)?

The last aspect is certainly the most critical (Hartung, 2007a). Most animal tests themselves have never been tested as to their relevance. Data from poison centers or clinical trials are only rarely available to compare with humans. However, we can carry out the same animal experiment with different species and ask, for example, how well do rats predict mice or hamsters predict guinea pigs? Obviously, there is no reason why any of these species should predict humans better than they predict each other. Many rodent species clearly are closer to each other than they are to humans. Even non-human primates have a certain evolutionary distance. The result is worrisome – the correlation between laboratory animal species usually only ranges between 60-70% (Hartung and Daston, 2009). What can we do? Traditionally, two paths are followed: One tests in two animal species, or one renders the tests precautionary, e.g., by testing extremely high dosages. Not “more is better” but “more kills better.”

5 How reliable are animal experiments?

Animal tests have made the world safer, but they also have created quite a few problems. We sort out more and more substances because of possible problems. The example of Aspirin is most interesting (Hartung, 2009a): Today aspirin would fail almost all safety tests. Aspirin kills half of the rats (LD₅₀) at doses we use as maximal allowed daily dose in humans. Today we typically request safety margins of a factor of 100, which means, in general, that we use doses that are at least 100 times smaller than those that harmed animals. Aspirin is an irritant to eye, skin, and lung. Aspirin has had ambiguous results in genotoxicity assays and, while not actually carcinogenic in the respective animal test, it augmented the carcinogenic effect of other substances when co-applied. Furthermore, aspirin led to embryonic malformations in practically every species tested (rats, mice, rabbits, cats, dogs, and monkeys). Note that these are all tests as they are used today for drugs, pesticides, and industrial chemicals. We know a lot about aspirin – 23,000 scientific publications are available, and a trillion (one thousand billion) tablets have been swallowed. None of the animal findings are really relevant for humans. But this shows that it would be impossible to bring aspirin to the market today. These, too,
are the costs of our desire for safety. This attitude is killing people as well—a new drug that is not allowed to go into the clinics because of alerts in precautionary animal tests is a drug that cannot cure patients.

At the same time, testing new substances in animals cannot prevent all dangers. When, after the respective animal tests, drugs are tested on volunteers and patients, 10-30% show toxic effects that will not allow developing them further (Kola and Landis, 2004). We simply are not 70 kg rats... There remains uncertainty on both sides—the false positive and false negative results. Animals represent only a model of humans, and all models are wrong, though some are still useful (Hartung, 2008a). It is most important that we are clear that we are using models that reflect only part of reality. Cell cultures (Hartung, 2007b) and computer models (Hartung and Hoffmann, 2009) have their own limitations. It is of utmost importance that we start analyzing the strengths and weaknesses of all our tools.

6 “Toxic Ignorance” – the REACH project and toxicity testing in the 21st century

Another problem is that testing in animals is far too expensive and laborious: To determine whether a substance is carcinogenic, for example, takes four years and costs about one million dollars. It is no surprise, then, that in the last 30 years in Europe only 14 of 5,000 new industrial chemicals were tested for their carcinogenic potential; out of more than 100,000 chemicals on the market, these represent only about 3,000. This has been termed “toxic ignorance” (Roe et al., 1997). The European REACH legislation aims to tackle this problem (Hartung, 2010a), but with traditional animal tests we will not achieve the throughput necessary (Hartung and Rovida, 2009; Rovida and Hartung, 2009). We simply do not have enough laboratories to test that many substances within a reasonable time frame. For this reason, REACH asks for new methods, but the implementation of the regulation is already foreseeable for the next decade. This leaves little room to develop and validate new approaches.

In addition to the ethical criticisms of animal tests, we must increasingly add a practical one: We cannot assess the safety of new substances coming to the market with sufficient certainty and speed (Hartung, 2009b). The renowned US National Academy of Sciences suggested in 2007 (http://www.nap.edu/catalog.php?record_id=11970) that toxicity testing in the 21st century has to move away from animal testing and establish a new safety testing paradigm. This has created an enormous atmosphere of departure. Currently, discussions are taking place at many venues regarding how to implement this (Collins et al., 2008; Hartung, 2009c; Firestone et al., 2010). Experts discuss a “Human Toxicology Project” (Seidle and Stephens, 2009), similar to the human genome project. We will see whether this can be financed. It promises to move the safety testing of products onto a new level, at least, but a lot of steering will be necessary (Hartung, 2009b). Most remarkably, the EPA already has made this their novel toxicity testing paradigm (Firestone et al., 2010). So we see very different approaches in the US and Europe: While the EU much earlier took up the challenge of old chemicals and only later aimed to reduce animal testing for animal welfare reasons, the US systematically developed a new approach based on new technologies although a testing program did not come about until now (Hartung, 2010b).

7 The technologies of the 21st century for the toxicology of the 21st century

What are the prospects and what are the new technologies? It has been claimed that knowledge in the life sciences doubles every seven years. In this case, we now have about 1,000 times more knowledge than was available at the time when most animal tests were devised. The revolutions in biotechnology and informatics we have seen occur not only on the stock market. Today, we have cell cultures for practically all tissues and organs of the human body. We know many pathways, we know how cells work, and we know how synthetic substances disturb these. Precise analytical methods, robotized testing, and complex measurements now allow enormous quantities of information to be obtained, and modern computers enable the analysis. The buzzword “systems toxicology” (Hartung et al., 2012) was coined to describe the systematic combination of existing knowledge via computer models with large datasets—from gene chips, for example—which can include all of the roughly 30,000 human genes (Hartung and Leist, 2008). This determines which genes in contact with a given poison are switched on or off. Similar poisons lead to similar responses (“signatures”). This also can be studied on the level of proteins produced or the changes in metabolite concentrations. Increasingly, we can deduce from this the pathways of toxicity that caused these molecular changes. The mapping of the entire pathways of toxicity, the human toxome, has been proposed (Hartung and McBride, 2011). Automated image analysis frequently plays a role, too. Still, a safety assessment that relies only on such methods and uses no animals remains a utopian vision. Twenty years ago, however, this held true for the mobile telephones we now take for granted, as well as today’s internet, which also was only emerging. In the developing laboratories we can already find the new toxicology techniques—they only have to be optimized to find their market.

And a market is there for sure: Each year industry spends about $3 billion on safety assessments worldwide (Bottini and Hartung, 2009). The European REACH program for old chemicals alone, which has just started will produce data costing $13 billion over the next ten years, and this is only the beginning: Nanoparticles, genetically modified food, cell therapies... new products lead to new challenges to control their risks (Hartung, 2010c; Hartung and Koeter, 2008). The job market for toxicologists is huge, and so it is good that some universities have again started to invest in their education.

Whether such a novel approach will improve how predictive toxicology is has yet to be seen. Everything starts, however, with no longer pretending how safe things are when they
enter the market. The pressure to develop a novel toxicology results only when the need for new technologies is clear. The use of animals is, in the end, a technology, the systematic use of a problem-solving approach. Blind faith in the meaningfulness of results from animal tests makes them an animal sacrifice for the invocation of a bright future for our products. A realistic judgment of their strengths and weaknesses, on the contrary, allows them to be used in a targeted manner to provide consumers with safe products at a more acceptable expense to animals. It also helps producers understand their safety gaps and toxicologists to develop new approaches allowing animal use to decline automatically. Because patients and consumers are of primary concern, the animals need not be secondary.

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Summary and Validation of New Animal-Free Toxicity Tests

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Summary
Alternatives to animal testing have been developed mainly in the fields of toxicology and vaccine testing. Typical examples are the evaluation of phototoxicity, eye irritation, or skin corrosion resulting from cosmetics and industrial chemicals. Examples also can be found in other biomedical areas, however, including the control of the quality of drug preparations or for the control of the production process of biologics. For regulatory purposes, the quality, transferability, and predictivity of an alternative method need to be evaluated. This procedure often is called the “validation process” of a new method. It follows defined rules, and several governmental institutions have been established to perform, supervise, or advise on this process. As this often results in a delay of method implementation, different alternatives for the evaluation of a method’s suitability and quality are subject to discussion. We describe here the principles of model development and quality control. We also give an overview of methods that have undergone validation. Strengths and shortcomings of traditional approaches are discussed, and new developments and challenges are outlined.

Keywords: validation process, OECD test guidelines, ICCVAM, ECVAM, JaCVAM

1 Introduction
Validation is a normal procedure in all fields of science once a test is developed (Hartung, 2007). The validation process is intended to provide confidence in the results, to define where the test may or may not be applied, and to give an account of test characteristics such as precision, accuracy, specificity, sensitivity, robustness, and transferability. The establishment, validation, and documentation of test methods in different areas of science all have been extensively covered in the specialist literature. This includes specific recommendations published by regulatory bodies. For instance, OECD GD 34 gives guidance on “Development, Validation and Regulatory Acceptance of New and Updated Internationally Acceptable Test Methods in Hazard Assessment.” While the predictivity and biological relevance often are difficult to quantify, the quality of an assay system may be assessed by strictly quantitative methods.

(a) Reproducibility: The experiment needs to be independent of the observer, the place, and the time when it is performed. That means that it should be repeatable by anyone (skilled in the art) and anywhere. The data should be quantifiable in order to establish reproducibility and comparability of the data.

(b) Relevance: The reason and rationale for the experiment should be clear and, most importantly, it should be embedded in a plausible biological context. This means, in a wider sense, that it is hypothesis-driven.

(c) Hypothesis-generating: The results of the experiment must point beyond the experiment itself and make predictions for other conditions. The predictions made must be testable and falsifiable.

These can be transferred to the requirements for model development, where three major criteria need to be fulfilled:

(aa) Reliability: This refers to the robustness and reproducibility of the model. Validation of this aspect should be mandatory for each model used, independent of the legal context or other implications. It is an evaluation of the technical quality of a model.

(bb) Translation: This refers to the scientific relevance of the model. Often judgments of this aspect will require that time and experience be gained through use of the model. Deep knowledge of biological processes involved in the model – and in reality – is required as well.

(cc) Predictivity: This aspect deals with the capacity of the model to yield results that correlate well with reality.
Specificity and sensitivity are among the parameters that describe this aspect. Notably, sensitivity and specificity are not only technical reliability parameters, as they change over time and with experience gained. Any given number is valid only in relation to the “gold standard” or the “reality” used as surrogate for reality. This point often is neglected or not recognized.

The model (i.e., the toxicological test used, e.g., as in vitro replacement method, or as animal model) itself is built from four elements. Each element can be validated and adapted individually:

(aaa) Biological system: This may be a dendritic cell or a guinea pig, or a differentiating stem cell.

(bbb) Exposure scheme: The guinea pig may be dosed orally or dermally, once or repeatedly, with a certain vehicle, for a certain time. The stem cell may be exposed to a chemical with or without medium change, during a certain time window and in a specified solvent.

(ccc) Assay endpoint: Death of a cell or of the guinea pig, measured by a specified viability assay, or using a specified humane endpoint; or skin reddening or altered differentiation, determined by PCR or immunocytochemistry. The type of endpoint chosen can completely change the outcome of an assay. In this context it is of utmost importance to distinguish endpoints that describe the biological system from endpoints that describe the behavior of the test in the presence of chemicals. These separate issues require independent optimization and characterization. For instance, a person’s body weight can be measured well on scales (to give a good readout on general growth characteristics of a person = biological system), but this endpoint will hardly respond to acute poisoning of the person. Instead, blood pressure or vomiting activity may be good measures of human poisoning (toxicological test), but they in turn give little information on the growth activity over time.

(ddd) Prediction model: Translation of an endpoint outcome into toxicological information. For instance: is reversible light skin reddening interpreted as a sign of sensitization? Or, is a change of gene expression of marker x interpreted as a toxicological change? Is there a binary outcome (toxic/non-toxic)? Or, are there more than two classes (mild, moderate, severe irritants, and how are the boundaries defined); if there are two or more assay endpoints, how are they combined into a final toxicity statement? During validation, the prediction model also needs scrutiny, and the questions asked are as follows: Is there a threshold (different from the statistical threshold) for when an effect can be considered biologically relevant? How is the outcome interpreted when more than one endpoint is measured (e.g., general cytotoxicity and functional impairment or effects on two different cell types)? Is an increase compared to normal good, when a decrease is bad? How should data be interpreted when a compound alters the baseline values for the endpoint (e.g., colored compound in spectrophotometric assays, reducing agents in tetrazolium reduction assays)?

Before validation of a method can be initiated, all the various scrutinized recently for its slow progress and potentially faulty outcomes, and alternatives are being considered. The evidence-based toxicology alternative has attempted to suggest alternative validation approaches (Hartung, 2010). It must be noted, however, that these require even more stringent definitions of the above criteria and of assay quality. Technical assay quality assessment is an indispensable step that should occur prior to any further validation steps addressing translation and predictivity.

3 Quality aspects of test systems

The description of a test system for regulatory purposes requires a standard operation procedure (Sesardic et al., 2004). This would, e.g., provide information on source and characterization of cells, a sufficient description of culture conditions for maintenance and experiment, and information on which parameters are critical and what affects them (Coecke et al., 2005). It also includes measurement methods, essential instrumentation, important manipulation steps, details on the determination of endpoints, and a description of the data processing.

Validation of model relevance needs to answer, for instance, the following questions: What human problem is modeled? What biological effect is it designed to measure? Which effects is the test designed to predict? Can it detect deviations from normal to both sides, or does the test work only for one side?

Important assay performance validation questions include: Does a compound that should change the endpoint do this – and by how much does it do this (=dynamics of the response, maximum possible deviation of endpoint); does a compound that is not expected to change the endpoint behave neutrally? It is frequently neglected, although scientifically important, that besides negative (NC) and positive (PC) controls (as above), many systems also require unspecific controls (UC). The response dynamics of a PC, and thus the performance of the test method, cannot be qualified without assessing the response to UC. It is important to re-challenge the test method with a new set of PC and NC (learning set, training set of chemicals) to assess its performance with respect to unknown compounds.

Frequently, test methods should assess specific adverse effects (SAE) independent of general cytotoxicity (GC). For instance, inhibition of neurite outgrowth can be measured meaningfully only in a concentration range that does not kill the cells (Kuegler et al., 2010). The toxicity range of test compounds may be determined as follows: a general cytotoxicity/viability test is run over a wide range of concentrations, initially with 10-fold dilutions. After identification of the relevant range, re-testing is performed in a more narrow range (3-fold dilutions) to identify the highest non-cytotoxic concentration (HNCC) within the conditions of the assay (e.g., a given time frame). For most practical purposes this may be done by using the mathematically defined IC₁₀ value of the cytotoxicity concentration response curve and moving to the left by a certain factor (e.g., HNCC = EC₁₀ x 0.02). Ideally, GC should be determined in parallel/simultaneously with SAE. Inability to measure GC does not
mean that it does not occur. This applies, in particular, to short-term assays (few hours), as most GC endpoints require several hours to become manifest.

Each experimental setup requires controls to indicate whether the experimental system reacts correctly, i.e., in the right direction, or in the right range. They give us an acceptance criterion for believing the other data obtained from unknown samples by the test method. The concept of acceptance criteria is highly important in all quantitative experimental sciences. Test systems, especially in in vitro toxicology, are usually so complex that they require known positive and negative controls to be measured along with the unknown samples (Leist et al., 2010). Only if these controls fulfill the acceptance criteria can the other experimental data be taken into consideration. Data from an experiment that did not fulfill the acceptance criteria cannot be used.

4 Controls and considerations required for the validation of assay predictivity

The predictive power usually is validated by examination of the correlation of assay results with a gold standard. However, correlation does not mean causality, even if the correlation is very good (Balls et al., 2006). On the one hand, the correlation may be real but exist only within a small range or under specific conditions or for a limited class of compounds. On the other hand, the correlation may not really exist but is suggested by the choice of compounds along the continuum of effects. This argument has an important practical implication for test compound selection. For instance, if the question is whether a simple 24 h fibroblast cytotoxicity assay correlates with a complex endpoint, such as chronic toxicity or carcinogenicity, it can be possible to find a good correlation if the 20 test compounds are comprised of 10 compounds of very low cytotoxicity and 10 compounds of high cytotoxicity. Some assays tend to agree when extremes are used, but the resulting (mathematically) good correlations may not hold true for test compounds in the intermediate range. Are such cases relevant and common? Yes, they are, particularly in studies using multiple endpoints. When dozens or hundreds of endpoints are used, such artificial correlations are likely to appear for at least some of them. Typical examples are -omics studies suggesting a correlation between some metabolite or protein modification with toxicity (Leist et al., 2008). For such studies, appropriate statistics use measures to counteract the effect of multiple endpoints on apparent significance of effects (false discovery rate corrections – FDR) (Benjamini et al., 2001).

The minimum information required on the response dynamics is the linear and dynamic range of the endpoint and the detection limit. Moreover, information should be provided regarding how stable (robust) a readout is. For instance, when neurite growth is measured, data are required on the length under optimal conditions (S) and on the variation of length under these conditions (V); in addition, the minimum length (N.B., this is not necessarily zero; it may, for example, be 50% of the maximum length measured in the presence of the strongest known growth inhibitor) that can be observed under the given assay conditions needs to be determined (B). Also, its variation (N) is an essential piece of information. From these data, the signal-noise ratio [S/N-ratio or (S-B)/N] can be calculated. These data also can be used to define the detection limit [e.g.: B + (5 x N)]. Another quality parameter of the test system (independent of any test compound) is the z’ factor, which, ideally, should be >0.5 and indicates the detection power of the system [z’ = 1 - ((3 x (V + N))/(S - B))]. The procedures used to determine z’ or S/N ratio also are well suited to detect systematic errors in the assay setup.

Toxicity curves do not necessarily follow a simple mathematical model, and they do not need to reach zero (viability) within the tested range of concentrations. For instance, only a subpopulation of cells may be affected. This means that EC50 values cannot be extrapolated. A meaningful EC50 requires that real data points (ideally ≥2) exist on both sides of the EC50.

5 Validation criteria and the validation process

The validation process itself has evolved over time to allow higher throughput, flexibility, and efficiency. For this, it is important to recall the main elements of an alternative method. As is evident, a test system is involved. This needs to be coupled with analysis endpoints and a data analysis procedure. Sometimes the third component is neglected: the prediction model relating the results of the method to predictions for human safety. A modular approach (Hartung et al., 2004) has been useful to accelerate the validation procedure. First, the reliability of the test system needs to be validated. This includes testing the descriptive assay parameters (accuracy, precision, detection limit, linear range, robustness, specificity, sensitivity, response dynamics) at increasing levels of complexity, i.e., within a laboratory (different operators) and between different laboratories (transferability). In parallel, the mechanistic validity and scientific relevance can be evaluated. In a third line of validation, the predictive capacity is evaluated. Until now, this has been done by correlation of the test results with the results of animal experiments. This process may yield information on applicability domains (e.g., only certain types of chemicals, but not others).

6 Validation by comparison with animal data

The field of alternative methods has tended to focus on one particular aspect of validation: the comparison to animal data. In this sense, validation and the phrases “valid methods” and “validated methods” have been used in legal texts, such as the European regulation on chemicals, REACH, the seventh amendment of the European Cosmetics Directive, and the new directive on the use and protection of experimental animals (2010/63EU). One of the consequences was the creation of a European validation agency in the field of toxicology, the European Centre for Validation of Alternative Methods.
(ECVAM) in Ispra (Italy). Comprehensive validation is a prerequisite for the adoption of a new method into a legal framework such as the OECD test guidelines or the European pharmacopoeia.

Validation in comparison with animal data has been criticized frequently. One argument is that animal experiments may not be suitable as a gold standard, as they do not correlate well enough with human data (Bahramsofani et al., 2009). Another argument is that such a correlative process is not possible when test batteries are used that do not model a defined animal experiment (Hartung, 2008). Therefore, new ideas have been proposed to overcome this problem. The most extreme approaches suggest neglecting the correlation aspects initially and, instead, focusing much more on the first two domains of validation: high quality of the test system and high scientific relevance which may, by themselves, provide a good predictivity for human safety. Such concepts, at present, are being tested and further developed with great speed.

The field of cosmetics is a good example for progress in the establishment and validation of alternative methods: replacement methods for some toxicological domains have been validated. These include phototoxicity, skin corrosion, skin irritation, eye corrosion, and eye irritation. Refinement/reduction methods also are available for acute oral toxicity (altered variants of the LD50 test) and skin sensitization (local lymph node assay) (ICCVAM, 2006). Many of these tests have been accepted by the OECD and, to a large extent, some have been substituted for the corresponding animal experiments.

According to current legislation, in 2013, animal testing for cosmetics has to stop in further toxicological domains. These domains include toxicokinetics, skin sensitization, repeated dose toxicity, carcinogenicity, and reproductive toxicity. A recent report published by the European Commission stated that sufficiently validated methods are not yet available in these domains. This opinion was confirmed by a large expert panel (Hartung et al., 2011) assembled by the Center for Alternatives to Animal Testing in Europe – CAAT-Europe (Daneshian et al., 2010). Thus, test development and validation are well under way, with high pressure in these domains.

7 Toxicological and other methods that have been validated

More than 80 methods have been validated or are in some more or less advanced state of validation. These include more than 50 in vitro tests, 10 using isolated organs, several refined in vivo tests, and testing strategies that combine in vitro and in vivo approaches. In vitro is defined as: “no animals are involved,” and the test is based on cell systems or isolated organs. Refined in vivo methods often involve the use of anesthetics and analgesics, and humane endpoints are applied. Furthermore, the development of tiered testing or testing strategies reduces the number of animals involved.

Many alternative methods are anchored in OECD (Organization for Economic Cooperation and Development) Guidelines. The guidelines for the testing of chemicals, as stated on the OECD website¹ “are a collection of about 100 of the most relevant internationally agreed testing methods used by government, industry, and independent laboratories to identify and characterize potential hazards of new and existing chemical substances, chemical preparations, and chemical mixtures. They are a basic set of tools used primarily in regulatory safety testing and subsequent chemical and chemical product notification and chemical registration. In addition, they also can be used for the selection and ranking of candidate chemicals during the development of new chemicals and products and in toxicology research.”

Another important source is the European Pharmacopoeia, and their mission is stated on their website²: “The texts of the European Pharmacopoeia (Ph. Eur.) concern the qualitative and quantitative composition of medicines, the tests to be carried out on medicines, on the raw materials used in the production of medicines and on the intermediates of synthesis. It contains texts covering substances, excipients and preparations for pharmaceutical use of chemical, animal, human or herbal origin, homoeopathic preparations and homoeopathic stocks, antibiotics, as well as dosage forms and containers. The texts also cover biologics, blood and plasma derivatives, vaccines and radiopharmaceutical preparations. They are legally binding.”

In the US, the Office of Chemical Safety and Pollution Prevention (OCBPP)³, under the umbrella of the Environmental Protection Agency (EPA), addresses the harmonization of chemical and pesticide testing.

Given that the area of test methods is under permanent development, it is rather challenging to keep track of the current situation. There are several sources available that try to document the status of 3R methods, but none can claim to be complete.

Information databases that may be consulted include:

A summary of the most prominent and widely accepted methods is provided below. The implementation of such assays in regular testing differs considerably between countries, institutions, and exact data requirements.

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¹ http://www.oecd-library.org/environment/oecd-guidelines-for-the-testing-of-chemicals-section-4-health-effects_20745788
³ http://www.epa.gov/aboutepa/ocspp.html
Acute aquatic toxicity
One validated test is anchored in the OECD TG 203\(^4\), using an upper threshold concentration (UTC) step-down approach, which reduces the number of fish used by 65\% (Hutchinson et al., 2003; Jeram et al., 2005; ECVAM, 2006b). Another test is under validation by ECVAM for an OECD Project to assess the transferability and reliability of the zebrafish embryo toxicity test for prediction of acute toxicity to fish. It is expected to be ready for implementation in 2012 (Selderslaghs et al., 2009, 2010).

Acute mammalian toxicity
Acute mammalian toxicity is divided into three subareas by their route of application. For the oral route, three tests have been validated and were implemented in the OECD TG 420\(^5\), 423\(^6\), 425\(^7\). All three methods reduce the number of animals used from 25 to 5-9 (van den Heuvel et al., 1990; Schlede et al., 1992, 1995; Diener and Schlede, 1999). The Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM) recommended two in vitro tests to be implemented into a tiered testing strategy to reduce the number of animals further (ICCVAM, 2001). Another test is under validation by ECVAM and is considered to be a follow-up validation study on the predictive capacity of the 3T3 Neutral Red Uptake cytotoxicity test to identify non-toxic substances for acute oral toxicity and its potential inclusion into an in vitro testing strategy for acute oral toxicity, which is expected to be finalized in 2012 (Stokes et al., 2006).

With respect to inhalation exposure, the original OECD TG 403\(^8\) is under revision to implement two validated tests that suggest humane endpoints and therefore are considered refinement methods.

One test is available for acute dermal toxicity, and it also applies humane endpoints. This may lead to a new OECD document (Draft TG 434).

Non-vaccine biologics
The Mouse LD\(_{50}\) Assay for Botox Potency Testing is a prominent example in the area of non-vaccine biologics. Eight alternative assays are available in different stages of regulatory acceptance. Some tests may have a large economic impact, as they are proprietary and implementation in a guideline would enforce their use by potential competitors. The Snap-25 test is listed as a method for replacement in the European Pharmacopeia for final batch testing (Ekong et al., 1997; Gaines Das et al., 1999; Sesardic et al., 2004), while two other assays are recognized by ICCVAM but further development is recommended (ICCVAM, 2008). There are three non-lethal mouse models, two listed in the European Pharmacopeia, and one is accepted only for BoNT type A (ICCVAM, 2008). Furthermore, there are two organ models; one is listed in the European Pharmacopeia (ICCVAM, 2008). The test for calcitonin bioactivity developed by Novartis is another example of an accepted alternative in the field of biologics (Hartung, 2001).

Vaccines
The testing of vaccines depends on their intended use, human or veterinary, and the testing addresses the vaccine’s potency or safety separately.

For vaccine potency in veterinary use, the lethal challenge test was replaced by an enzyme-linked immunosorbent assay (ELISA), a biochemical analytical approach. It is implemented in the European Pharmacopeia, e.g., swine erysipelas vaccine (Pastoret et al., 2002; Rosskopf-Streicher et al., 1999, 2001).

In testing the vaccine potency for human use, seven tests are implemented in the European Pharmacopeia. The lethal paralysis challenge test for batch potency of tetanus toxoid vaccines may be replaced by an ELISA measurement (Balls and Hellsten, 2000b) and a toxin binding inhibition method (Balls and Hellsten, 2000a); the diphtheria vaccine may be tested via a cell-based assay and an ELISA (Council of Europe, 2008). Hepatitis B and Poliomyelitis vaccines are tested via serological antigen quantification (Council of Europe, 2008), and Rabies potency testing is done by using only one dilution, and humane endpoints are applied (Council of Europe, 2008).

The formerly used target animal vaccine safety test for veterinary use could be dropped as a result of a retrospective study conducted by ECVAM.

In the area of vaccine safety for human use, the following four tests are available: i) the abnormal toxicity test can be deleted from the testing scheme when batch consistency can be demonstrated (Schwanig et al., 1997); ii) the oral polio neurovirulence test conducted in monkeys may be replaced by an in vitro test called “MAPREC,” but only for type 3 oral polio virus vaccines (WHO, 2005); iii) the use of transgenic mice instead of monkeys (TgPVR21) was validated by the World Health Organization (WHO) for type 1,2,3 oral polio vaccines (Dragunsky et al., 2003); and iv) the residual toxicity of diphtheria may be replaced by the Vero Cell Test (Council of Europe, 2008).

Chronic toxicity
In the area of chronic toxicity for pesticides, the 1-year dog study was found to be unnecessary based on a statement of the ECVAM Scientific Advisory Committee (ESAC) and the US Environmental Protection Agency (EPA). It was found that the 1-year study does not provide more information than the 90 day study, but some countries still require these data (OECD, 1998).

Eye corrosion and irritation
For eye corrosion and irritation studies ICCVAM will implement the routine use of anesthetics, systemic analgesics and humane

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\(^4\) http://www.oecd.org/dataoecd/17/20/1948241.pdf  
\(^7\) http://www.oecd.org/dataoecd/17/5/1948378.pdf  
\(^8\) http://www.oecd.org/dataoecd/64/55/41761261.pdf
endpoints, as well as several validated tests, laid down in the OECD TG 437\(^9\) and 438\(^9\), and an OECD GD supplement.

Furthermore, there is one test under validation by ECVAM and the European Cosmetics Association (COLIPA) to assess the transferability, reliability, and predictive capacity of two in vitro test methods based on reconstructed human tissue models, to be used as stand-alone test methods to identify chemicals not classified as eye irritant (non-irritant) (McNamee et al., 2007).

**Food safety**
In the area of food safety, two tests have been validated to replace the Mouse Bioassay for shellfish toxins (PSP). One screening method and a high performance liquid chromatography (HPLC) approach were accepted in the EU in 2010 (Jellett et al., 2002; Mackintosh et al., 2002; FAO/IOC/WHO, 2004).

**Carcinogenicity**
There are two tests under validation for carcinogenicity to assess protocol standardization, transferability, and reproducibility (but not performance) of three protocols of cell transformation assays: the Syrian hamster embryo (SHE) pH 6.7, the Syrian hamster embryo (SHE) pH 7.0, and the BALB/c 3T3 assays. Furthermore, a validation study is underway to verify if the Bhas 42 cells-based cell transformation assay might be an equivalent (Combes et al., 1999; Maurici et al., 2005; OECD, 2007). Results are expected in 2012.

**Genotoxicity**
The area of genotoxicity is covered by eight validated in vitro tests, which are part of a tiered testing strategy to reduce the number of animals. They are reflected in several OECD documents, and two in vitro comet assays are under validation (OECD, 1986a,b,c,d; OECD, 1997a,b,c).

**Hematotoxicity**
One hematotoxicity test for acute neutropenia (CFU-GM) has been validated by ECVAM. The test can be applied instead of a second animal species. Therefore, it is not considered a replacement, though a reduction in the number of animals is achieved (Pessina et al., 2003).

**Phototoxicity**
To determine phototoxicity, the European Commission accepted the in vitro neutral red uptake (NRU) phototoxicity test (OECD, 2004a) as method B.41 in Annex V of the EU Council Directive 67/548/EEC\(^11\). Animal methods to detect phototoxic effects of chemicals are prohibited in all Member States.

**Pyrogenicity**
To replace the rabbit pyrogen test, five in vitro tests based on human cell models have been validated by ECVAM (ECVAM, 2006c). They can be used to detect gram-negative mediated pyrogenicity. The official European Pharmacopoeia listed test, the Limulus amoebocyte lysate assay (LAL), lacks the capability of detecting gram-positive stimuli. The cell-based assays also may be useful for gram-positive mediated pyrogenicity (ECVAM, 2006c; NICEATM-ICCVAM, 2007, 2008, 2009; Poole et al., 2003; Hoffmann et al., 2005a,b). This might lead to a full replacement of the rabbit test in the near future.

**Reproductive and developmental toxicity**
Due to the complexity of the reproductive cycle and the importance of the developmental process, not many alternatives are available in these areas. The OECD only recently accepted the extended one-generation study (OECD, 2008), which replaces the two-generation study (OECD, 1983). Furthermore, as stated in OECD TG 415\(^12\); “For reproductive endpoints, it is envisaged that, as a first step and when available, information from repeat-dose studies (including screening reproductive toxicity studies, e.g., TG 422), or short term endocrine disrupter screening assays (e.g., Uterotrophic assay – TG 440; and Hershberger assay – TG 441) are used to detect effects on reproductive organs for males and females. This might include spermatogenesis (testicular histopathology) for males and estrous cycles, follicle counts/oocyte maturation, and ovarian integrity (histopathology) for females. The Extended One-Generation Reproductive Toxicity Study then serves as a test for reproductive endpoints that require the interaction of males with females, females with conceptus, and females with offspring and the F1 generation until after sexual maturity.”

There are also two ECVAM-validated in vitro tests using embryos from animals (ECVAM, 2006a; Spielmann et al., 2006). In addition, there is one stem cell-based test (EST) available (ECVAM, 2002), which ECVAM recommended to be part of a tiered testing strategy.

**Endocrine active substances**
There are two OECD accepted methods, anchored in the OECD TG 455\(^13\) and 456\(^14\); they may be used for screening purposes. The US EPA accepted a Tier 1 screening battery including, besides several in vivo assays, five in vitro tests that have been accepted by the Office of Prevention, Pesticides and Toxic Substances (OPPTS) and laid down as legally binding guidelines for the US as Series 890 OPPTS\(^15\).

There are two methods under validation by ICCVAM, ECVAM, and the Japanese Centre for the Validation of Alternative Methods (JaCVAM) to assess the transferability and reliability of the assays to rank chemicals according to their potency for estrogen

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\(^10\) http://ecvam.jrc.it/ft_doc/OECD%20TG%20438.pdf
\(^11\) http://eur-lex.europa.eu/LexUriServ/LexUriServ.
\(^12\) http://www.oecd.org/dataoecd/23/10/48466062.pdf
\(^14\) http://bit.ly/7iwgDK
\(^15\) http://www.epa.gov/ocspp/pubs/frs/publications/Test_Guidelines/series890.htm
receptor activation or suppression for use as a building block in future testing strategies to detect endocrine active compounds. Evaluation is expected to be finished in 2012.

Skin
There are many tests available for hazard assessment regarding the human skin. They are divided into four different areas: absorption, penetration, corrosion, and irritation.

For skin absorption and dermal penetration, a regulatory accepted dermal percutaneous test is available, which may replace the animal test when human skin is used (OECD, 2004b). More information is available in the OECD guidance document16.

For skin corrosion, three different methods are available, all integrated in OECD guidelines (OECD, 2004c, d, 2006), but their use is specified. For example, the test “Corrositex” can be used to identify acids and bases, and substances that are identified as corrosives will not proceed further to the animal test. The “TER” test can distinguish between corrosives and non-corrosives, but non-corrosives will require further confirmation by an animal test. The human skin models (EpiSkin™, EpiDerm™, SkinEthic™) are accepted in the EU as full replacements for corrosivity testing anchored in Regulation 440/2008/EC. In the US these tests can be used to exclude corrosives, while negative results lead to an animal test.

Skin irritation can be detected via the above mentioned human skin models. Tests may be adopted in a new OECD draft guideline and may be used to identify irritants.

Dermal sensitization
Sensitization is detected by local lymph node in vivo assay (LLNA) which is recommended by ICCVAM as a stand-alone substitute for the guinea-pig sensitization test (OECD, 2002; ICCVAM, 1999; ISO, 2002; EPA, 2003; ECVM, 2000). The reduced rLLNA is able to distinguish between sensitizers and non-sensitizers, and if a chemical is negative in the rLLNA, it will not proceed to the full LLNA, which results in the use of fewer animals (ECVM, 2007). There are three methods under validation by ECVM, ICCVAM, and JaCVAM to assess the assay’s transferability and reliability in view of future incorporation into a testing strategy for full replacement of current regulatory animal tests. COLIPA, the EU project Sens-it-iv, and many others have developed a whole battery of pure in vitro assays that potentially could lead to a full animal replacement within a few years.

Toxicokinetics and metabolism
The area of toxicokinetics and metabolism is considered to be very complex and difficult to model. Nevertheless, two assays are under validation by ECVM, ICCVAM, and JaCVAM to assess the transferability and reliability of measuring liver enzyme (Cytochrome P450) induction using the human cryoHepaRG® cell line and cryopreserved human hepatocytes to provide a human metabolically-competent model for use in future testing.

For the field of kinetics testing, as for sensitization, carcinogenicity, toxicokinetics, repeat dose toxicity, and reproductive toxicity, the status of replacement methods has been reviewed extensively (Adler et al., 2011; Hartung et al., 2011).

8 Old versus new approaches to validation
Validation approaches are closely linked to the concept initially used for model development. In this context, it is important to recall that there are two fundamentally different ways of constructing test systems, which we call here (a) “correlative approach,” and (b) “re-constructive approach.”

(a) Correlative approach
The correlative approach has been used most frequently for the establishment of alternative methods for animal experimentation and, therefore, the whole theoretical concept of “validation” has been adapted to this approach. In brief, this approach uses the test method as input-output system. Validation in this case is largely concerned with an evaluation of how well input correlates with output. The model itself often is a kind of black box, with only limited information available on the relevant processes and reactions inside. This has the key advantage that reasonable correlations can be obtained without the need for knowledge on mechanisms of toxicity, or of regulatory mechanisms within the model. The disadvantage, obviously, is that the relevant processes often are not known.

Examples illustrate this situation best. The first is the mouse cancer bioassay. When it was established, it was a true black box model. The rationale was only that some correlation was expected between compounds known to be carcinogenic in man and the ones triggering tumorigenesis in mice. Mechanisms of carcinogenesis were largely unknown and did not necessarily need to be known for this model. Very powerful carcinogens and clear non-carcinogens correlated nicely between this model and the situation in man. Problems became obvious when a lack of correlation was observed for several classes of compounds. For instance, the so-called peroxisome proliferators triggered hepatocarcinogenesis in the mouse but not in man.

Another example from the same field of toxicology is the Ames bacterial mutagenesis assay, which was introduced to detect mutagens, at that time believed to be carcinogens as well. Back-mutation of errors in the bacterial genes coding for histidine synthesis obviously have no resemblance or biological relevance with respect to human carcinogenesis, but the model achieved a reasonably good overall correlation and therefore was widely accepted. It only became evident later that about half of the human carcinogens are non-mutagens and, therefore, cannot be detected in this assay. A correction or adaptation of the assay is not possible, as it does not reflect human biology. It was established simply as a correlative black-box model.

A third example also comes from the field of carcinogenesis. The human cell transformation assay predicts mutagenic and

16 http://www.oecd.org/dataoecd/0/1/46257610.pdf
epigenetic carcinogens with an astonishingly high specificity. It is still unclear why the assay works and what the underlying biological principle is. On the basis of correlations with human or animal data, however, the validation of the assay is far advanced.

The strength of this correlative model setup is proven by the assays that have been developed on this basis and have been successfully validated and used. Model development was possible without the requirement for in-depth biological knowledge. The weaknesses are demonstrated on the example of the embryonic stem cell test (EST). The EST uses murine embryonic stem cells (EST). They are differentiated with a very rough protocol to mixed cultures containing cardiomyocytes and pacemaker cells, resulting in patches of cells that beat spontaneously. Compounds are being tested for their potential to inhibit the development of these beating cell clumps. In initial validations, the assay was found to predict teratogens with high specificity and sensitivity, and it was recommended by ECVAM and the ECVAM Scientific Advisory Committee (ESAC) for regulatory use. A biological characterization had never taken place, and the mechanisms and regulations underlying this assay were never characterized. The use of a small number of validation compounds and the absence of biological knowledge harbors some dangers, as demonstrated by the history of this assay. In a broader validation with compounds chosen within the context of the ReProTect study, the assay failed (Marx-Stoelting et al., 2009).

A priori, it may not seem necessary to understand an assay as long as it delivers good (= predictive and reproducible) results. Toxicological testing has largely adopted this approach, not just in vivo, but also in vitro. However, there are strong reasons to move ahead to mechanism-based in vitro tests to attribute a scientific rationale to the correlations found in new test systems. Paradoxically, modern technologies are especially likely to settle for black box approaches and blind correlations. Such approaches bear the risk of measuring trivialities if they are not based on a mechanistic rationale. For example: new metabolomic or transcriptomic fingerprints to predict complex forms of toxicity (e.g., developmental toxicity) may indeed only be expensive and sensitive measures of classical cytotoxicity. Results only gain scientific validity when they are controlled by various approaches and when falsification attempts of their predictions have failed.

(b) Re-constructive approach
The second type of modeling was termed the “re-constructive approach.” This name was chosen because such models try to reconstruct reality using biological information and mathematical relationships between model parameters as building blocks. This approach requires an understanding of the biological process to be modeled, not only in qualitative, but also in quantitative terms. A biological process needs to be dissected into all its components. Each component needs to be understood. Moreover, the relationships between the components need to be understood, and mathematical approaches need to be developed to describe the relationship between all components and parameters. Finally, these elements can be used for “re-constructing” reality as closely as possible. An example is physiology-based pharmacokinetic (PBPK) modeling. The corresponding black box model is the injection of compounds into animals and the evaluation of their pharmacokinetic behavior (time course of plasma concentrations, urinary excretion …) and the correlation of this information with the expected behavior in man. PBPK modeling would use information on hepatic metabolism, solubility, lipophilicity, and renal excretion to model the behavior of the drug in a human body, using a set of differential equations. The validation of such models would refer not only to the input-output correlation but also to the construct of the model. This is a difficult task, and firm guidelines for this have not yet been established.

It is noteworthy that, in reality, the two extremes of black box modeling and pure reconstruction barely exist. Often, the approaches are combined to some extent. For instance, PBPK models would use information obtained from rodent models. Then information would be used from human and rodent liver metabolism, and this information then would be used to translate rodent information better into human information in an optimized PBPK model using the so-called parallelogram approach. Other examples, below, illustrate the incorporation of biological and mechanistic information into correlative models. For instance, in the case of skin irritation, originally the damage to skin was measured by classical viability assays. Attempts to account for inflammatory processes and active reactions of cells in the skin by measurement of chemokine release are ongoing. Also, in the field of sensitization, biological information is incorporated into available models. One approach, for instance, tested the effect of keratinocyte addition in a dendritic cell activation model to reflect their normal biological presence and role in co-stimulation and haptenization.

The validation of integrated testing strategies provides new dimensions of challenges. This will somehow require the validation not only of individual components but also of the relationships established between them and used for the overall modeling. Thus, this form of validation combines issues from the two types of model validation, correlative and re-constructive, discussed above. The challenges of such an approach may be illustrated by the example of dermal sensitization. An integrated test battery may involve a haptenation assay, measuring the covalent binding of the chemical to a peptide. It also may involve some physicochemical characterization to be used to predict skin penetration. A dendritic cell activation assay, in the absence or presence of keratinocytes, would be added. Eventually, T cell stimulation may be probed as well. Then the test strategy parts will have to be linked and weighted. One simple rule may be: if a compound is positive in one of the assays, it is considered a sensitizer. More complex sets of rules would use a hierarchical decision setup. For instance, compounds unlikely to penetrate the skin or without chemical reactivity may be considered of low hazard, even in the presence of positive dendritic cell activation. Such integrated testing strategies (ITS) may again
be validated by a correlative approach of the overall ITS versus reality. This most likely will be the first and most immediate solution in the near future. Re-constructive validations of such approaches will require huge amounts of data and experience, but they may become necessary in cases in which little “reality” information is available, such as the area of developmental neurotoxicity.

9 Outlook on validation in the field of developmental neurotoxicity (DNT)

Developmental neurotoxicity is an area that requires such new validation concepts, as not enough animal data are available. A recent review revealed that just over 100 compounds have been tested in studies using the OECD 426 draft guideline on developmental neurotoxicity. Most of these compounds were pesticides (66%); only eight industrial chemicals were included. Another review identified 174 compounds for which neurobehavioral risk assessment had been performed, in many cases on the offspring of the exposed animals (F1 generation) as well. Only 1% of these compounds were industrial chemicals, and thus the available data regarding the developmental neurotoxicity of industrial chemicals is rather limited. For some compounds, developmental neurotoxicity is the most sensitive of all toxicity endpoints evaluated in a broad safety evaluation battery. Thus, although developmental neurotoxicity appears to be an important domain of safety evaluation, test capacity is limited and test costs are extremely high. This puts pressure on the development of faster and cheaper in vitro systems that can predict developmental neurotoxicity, give information comparable of behavioral readouts, and facilitate screening, or at least prioritization of relevant drugs and chemicals for further testing. We envision that future in vitro test systems for developmental neurotoxicity will combine the above validation approaches with exposure information, and we suggest a strategy for test system development and cell-based risk assessment.

We propose that the emerging knowledge from molecular and cellular neuroscience and mechanistic neurotoxicology can be exploited to design in vitro tests that read out cellular and molecular endpoints that are predictive of behavioral signs of neurotoxic exposures in humans. For acute or more chronic neurotoxic effects, the onset of these effects is temporally associated with the onset of the chemical exposure and usually follows a dose-response relationship. The discipline of developmental/neurodevelopmental toxicology faces an additional problem, however. It is difficult to provide evidence for cause-effect relationships for processes with a long time lag between exposure (e.g., gestational) and effect (e.g., adult life). Suitable test systems for delayed effects need to be identified. Research in this particular area is motivated by increasing incidence of neurodevelopmental disorders such as autism, ADHD, and schizophrenia, as well as the growing awareness that environmental factors influence susceptibility to and/or severity of these diseases.

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New Paradigm in Toxicity Testing: 
Integrated Discrete Multiple Organ Co-cultures (IdMOC) 
for the Evaluation of Xenobiotic Toxicity

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Summary
Humans are exposed, intentionally or unintentionally, to a number of novel chemicals due to industrial activities. There is an urgent need to evaluate the biological effects of these novel chemicals efficiently and accurately. The Tox21 and ToxCast™ programs initiated by the National Institutes of Health (NIH) and the EPA recommend the use of innovative chemical testing methods to meet this unprecedented demand. The critical paths initiative of the U.S. FDA emphasizes experimental approaches that are directly translatable to clinical observations. Application of high-throughput, in vitro assays to delineate pharmacological and toxicological pathways in combination with computational modeling represents one of the critical strategies to predict chemical effects on animals and humans. The Integrated Discrete Multiple Organ Co-culture (IdMOC) is a novel in vitro experimental system that allows the evaluation of biological effects of chemicals, with interactions between multiple cell types including endocrine, paracrine, and metabolic interactions. One specific application of IdMOC is the evaluation of metabolism-dependent chemical properties such as metabolism-dependent toxicity and pharmacology.

Keywords: co-culture, IdMOC, ToxCast, Tox21, in vitro toxicity testing

1 Introduction

Concerns regarding the toxicity of environmental chemicals, consumer products, food additives, pharmaceuticals, and biologics have prompted several US legislative and regulatory initiatives. In 1998, the U.S. Environmental Protection Agency (EPA) initiated the High Production Volume (HPV) program\(^1\) to provide health and environmental data for chemicals that are manufactured or imported in the United States in quantities greater than 1 million pounds annually. This represented 95% of US chemical production and use by volume at the time. The extended HPV program\(^2\) was announced in 2005 to address newer HPV chemicals (574) and broaden the scope of the program to include use and exposure information of all HPV chemicals. The European Union passed the REACH law (Registration, Evaluation, Authorization and restriction of Chemicals), which aimed to regulate all chemicals produced in quantities greater than one ton/year. In 2009, the EPA proposed a set of 73 chemicals for initial screening, followed by another set in 2010 consisting of 134 chemicals (Borgert, et al., 2011). In addition to the unprecedented demands of REACH and the EPA, the National Toxicology Program (NTP), based at the National Institutes of Health and Environmental Sciences (NIEHS), has actively solicited nominations from the public and scientific community for toxicological evaluation of chemicals\(^3\). Such nominations are reviewed based on supporting information and priorities of the agency and, when considered appropriate, are evaluated at the laboratories of the NIEHS. The U.S. Food and Drug Administration (FDA) has routinely regulated pharmaceuticals, biologics (vaccines, blood products), devices, food additives, veterinary products, and cosmetics for safety. Thus, there is a clear need for increased testing and early screening of thousands of compounds to eliminate dangerous substances and develop safe products with marketable potential.

\(^1\) http://www.epa.gov/chemrtk/
\(^2\) http://www.americanchemistry.com/Policy/Chemical-Safety/High-Production-Volume
\(^3\) http://ntp.niehs.nih.gov/?objectid=25BC6AF8-BDB7-CEBA-F18554656CC4FCD9
2 The challenge

The scientific basis and corresponding methodologies for hazard identification and risk assessment must be modified such that an increasing number of chemicals can be evaluated in a short period of time. This has been recognized recently by U.S. federal and regulatory agencies. In 2008, a partnership was announced between the EPA, NIEHS/NTP, NIH Chemical Genomics Center, and the Food and Drug Administration (FDA) to meet the future toxicity testing and risk assessment needs of the country. This initiative, termed Tox21, recommended implementation of the National Research Council Committee’s plan, *Toxicity Testing in the 21st Century: A Vision and a Strategy* (NRC, 2007). The plan proposed the development of innovative chemical testing methods that characterize chemically induced toxicity pathways. The program aimed to identify chemically induced biological mechanisms, prioritize chemicals that require further toxicological evaluation, and develop models that effectively predict how chemicals will affect such biological responses. It recommended exploration of high-throughput screening assays, tests using phyleogenically lower animal species (e.g., fish, worms, etc.), and high-throughput whole genome analytical methods to evaluate biological mechanisms. Hence, these tests are designed to predict toxicological endpoints (e.g., toxic dose) from a cell-based response using pharmacokinetic and computational modeling (Firestone et al., 2010; Kavlock et al., 2009; Locke and Bruce Myers, 2010). While such methodologies are indeed rapid and promising, they rely ultimately on computerized predictions (Kavlock and Dix, 2010). The predictive analysis of new chemicals based on information from previously examined chemicals, however, is dependent upon similarity to the reference chemicals used. If the effect of a reference chemical is unanticipated, e.g., bioactivation of the parent compound into a more toxic metabolite, then similar effects of the test material are likely to remain undiscovered (Schmidt, 2009).

In 2007, the EPA independently launched the ToxCast™ program to develop methods to predict potential toxicity and, based on such predictions, to prioritize the thousands of chemicals that need toxicity testing (Dix et al., 2007). Since then, the EPA has profiled approximately 300 chemicals, primarily pesticides, using high-throughput in vitro assays. Dose response relationships evaluating specific endpoints such as gene expression and receptor binding ability have been reported (Knudsen et al., 2011; Rotroff et al., 2010). The EPA has also catalogued traditional toxicity data obtained from animal studies for many of these chemicals (ToxRefDB5). The challenge here is to validate the predictive signatures and data so that this endeavor can be translated into a program to successfully screen new compounds for determination of toxicity.

3 Single cell type in vitro toxicity and metabolism assays

Two major considerations or research gaps identified in the development of toxicity assays and the identification of pathways include the incorporation of metabolism (hepatic and non-hepatic) into in vitro assays and the means to study cell-cell interactions (Schmidt, 2009; Judson et al., 2010; Memorandum of Understanding on High Throughput Screening6). Currently, 2-dimensional monolayers of mammalian cells provide the most suitable in vitro model. This is due largely to the fact that such models are conducive to repetitive, systematic, and quantitative evaluation of the biological response to a chemical insult. In vitro assays also are more cost-effective, less time consuming, and can assess a large number of compounds and experimental parameters compared to in vivo assays (Knight, 2008).

3.1 Hepatic metabolism assays

The liver is the central organ for xenobiotic metabolism. All ingested drugs and chemicals undergo a first pass through the liver after intestinal absorption. The hepatocytes in the liver contain various drug metabolizing enzymes, including phase I oxidative enzymes (e.g., CYP 450 dependent monoxygenases) and phase II conjugating enzymes (e.g., UDP-glucuronosyltransferases, Glutathione S-transferases), as well as drug transporters that contribute to the metabolism and disposition of xenobiotics. Thus, it is not surprising that primary hepatocyte cultures generally are regarded as the gold standard for assessing xenobiotic metabolism. Primary hepatocytes derived from cadaver or transplantable human livers provide the most physiologically relevant experimental system, since they are capable of human xenobiotic metabolism. As such, they provide human-specific information for the prediction of xenobiotic toxicity (Li, 2007a). Primary human hepatocytes can be used for studying various drug properties, including metabolic fate, drug-drug interaction potential, and hepatotoxicity. The development of advanced cryopreservation techniques to prevent membrane damage and subsequent lysis during thawing, along with the development of specific hepatocyte media, has facilitated widespread use of these cells in toxicity assays. Monolayers of hepatocytes at 80-95% confluency are routinely cultured after immediate recovery from cryopreservation and can be used to test drugs and other chemicals effectively. Treatment periods of 24-48 hours are well suited to the development of rapid toxicity tests and can be used in the early assessment of hepatotoxicity and xenobiotic metabolism (Li, 2007a; Hewitt et al., 2007; Li et al., 1999a).

The cellular microenvironment is the key to the physiological response to drugs, as it can affect the cell’s properties, behavior, and function. Interaction of a cell with the extracellular matrix (ECM) or adhesion molecules can trigger a cascade of molecular

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5 http://www.epa.gov/nct/toxrefdb/18
events within the cell that culminate in specific cellular responses such as proliferation, differentiation, metabolism, or morphogenesis (Elliott and Yuan, 2011). To model appropriate cellular responses, monolayers of hepatocytes are often sandwiched between two layers of the ECM, namely collagen at the bottom and matrigel or collagen on the top. Using this method, Kern et al. showed that metabolic activity in response to the inducer rifampin could be maintained for 9 days and was comparable to that observed in vivo (Kern et al., 1997). Kienhuis et al. compared sandwich cultures of rat hepatocytes in media with or without low doses of the P450 inducers-phenobarbital, dexamethasone, and β-naphthoflavone and found that coumarin-induced cytotoxicity, metabolism, and gene expression was more similar to the in vivo situation using modified media than to the control media (Kienhuis et al., 2006). The presence of soluble factors such as growth and survival factors, differentiating agents, and inflammatory mediators, also can affect the competency of a cell. Hence, specific factors such as insulin and dexamethasone often are added to increase survival and maintain the differentiated functions of hepatocytes in culture.

Human hepatocytes represent a valuable experimental system for drug metabolism studies. Hepatocytes can be used in screening assays for metabolic stability and metabolite identification, and demonstration of key metabolizing pathways, as well as evaluation of P450 enzyme activity. Unlike cell-free systems such as microsomes, intact hepatocytes contain all the drug metabolizing enzymes and cofactors at physiological levels. Microsomes also contain only phase I oxidizing enzymes, so unless Phase I oxidation is the most important metabolizing pathway of the test material, intact hepatocytes provide a more physiologically relevant model (Li, 2007a). Metabolite profiling of the drug ethynyl estradiol in the presence of microsomes revealed 2-hydroxylated derivative as the predominant metabolite, whereas profiling in hepatocytes revealed glucuronide and sulfate conjugates similar to those seen in in vivo studies (Li et al., 1999b).

3.2 Non-hepatic organ-specific metabolism assays

Other cell types besides hepatocytes also have metabolizing abilities. For example, astrocytes from the central nervous system express monooamine oxidase B that can metabolize the drug MPTP in the brain (Brooks et al., 1989; Gerlach et al., 1991). In addition, several cell types contain CYP enzymes that may be induced by certain drugs. CYP1A1 is inducible in the epidermis in response to TCDD (tetrachlorodibenzo-p-dioxin), PCBs (polychlorinated biphenyls) and crude coal tar (Walsh et al., 1992). CYP1B, CYP4A, and CYP2E1 are induced while CYP2B1 and CYP2C11 are inhibited by doxorubicin in the kidney (Zordok et al., 2011). Hence, metabolism assays using organ-specific target cells capable of metabolizing chemicals are vital in the evaluation of cytotoxicity.

In some organ-specific tissues such as the lung, several P450 enzymes including CYP1A1, CYP1B1, CYP2A6, CYP2B6, CYP2E1, CYP3A5, and Phase II enzymes such as epoxide hydrolase, UGT1A (glucuronyl transferase), as well as GST-P1 (glutathione S-transferase), have been detected (Castell et al., 2005; Mace et al., 1998). However, the key question is whether cells derived from such tissues have the capacity to activate or detoxify chemicals. Since metabolic activity typically diminishes on plating or culture of cells, it becomes imperative to rigorously test all primary cultures and cell lines for metabolic competence. Genetic manipulation of organ-derived cells to re-express key biotransformation enzymes has been suggested as a means to improve their functionality and metabolic performance (Castell et al., 2005).

Hepatic metabolism can lead to detoxification of compounds or biotransformation into highly reactive and toxic metabolites. Bioactivation of pro-carcinogens, i.e., polycyclic aromatic hydrocarbons or N-nitrosamines into reactive intermediates that can form DNA adducts has been shown to occur in the lung (Uppstad et al., 2010; Lao et al., 2007). Current evidence suggests that ifosfamide metabolites, particularly chloroacetaldehyde, produced within the kidney, contribute to nephrotoxicity (Springate and Taub, 2007). While the choice of the metabolically active cell is critical, the target cell may be distinct from the metabolically competent cell type. For example, astrocytes in the substantia nigra of the brain are capable of metabolizing MPTP into the active metabolite MPP+. MPP+ has been shown to cause loss of dopaminergic neurons (Brooks et al., 1989), suggesting induced neurotoxicity in the presence of metabolically competent astrocytes. Similarly, certain organophosphates such as isofenphos can be converted into potent toxic oxon metabolites by hepatocytes. These, in turn, can affect neuronal acetylcholinesterase inhibition (Bruinink and Maier, 2007), suggesting a role for hepatocytes in potentiating neurotoxicity.

4 Multiple cell type metabolism and toxicity assays

The use of single cell-type cultures for evaluation of organ-specific toxicity is limiting since such systems ignore the critical interactions between different cell types within an organ or between multiple organs. Co-cultures of rat hepatocytes with stellate cells in spheroidal aggregates produce higher enzymatic activities than monocultures of hepatocytes. This was evidenced by a significant increase in P450-mediated metabolism of testosterone in coculture compared to monoculture. These studies highlight the importance of parenchymal-stromal cell interactions in retaining metabolic function (Thomas et al., 2006).

Isolated organ cultures, including liver spheroids and artificial liver constructs, mimic some of the histological features of the liver as well as permit interactions between different cell types normally present in the liver. Liver slices, consisting of multiple differentiated cell types also provide simulated cell-cell and cell-matrix interactions. However cellular viability is difficult to maintain in liver slices and is restricted to organ-specific interactions (Knight, 2008).

Toxic metabolites produced in the liver constitute a significant proportion of all toxins. For most compounds, however, the degree of toxicity resulting from metabolic activation is unknown.
material, and individual cell types can be processed for the quantification of associated test material and specific biologic responses such as proliferation, apoptosis, cell cycle arrest, gene expression, and cytokine production.

The IdMOC model is a simple experimental system structured in a 6, 24, or 96-well format and hence is conducive to high throughput techniques. It can be integrated with most common assay platforms that use fluorescence, luminescence, and histochemical analyses, and do not require any specialized equipment (Li, 2008). Applications of this system are highlighted below.

### 5.1 Detection of organ-selective toxicity with IdMOC

Developed for the evaluation of xenobiotic toxicity, this model uses a wells-in-a-well concept (Li, 2008). The IdMOC system consists of a unique cell culture plate that contains multiple inner wells embedded in an outer, larger chamber (see Fig. 1). Multiple cell types are first individually cultured in the inner wells using media optimized for each cell type. An advantage of this system is that individual cell types, such as hepatocytes, can be cultured in a sandwich configuration with selective media for optimal expression and activity of metabolizing enzymes. On the day of experimentation, the individual media are removed and the outer chamber is filled with a proprietary universal medium. This floods the inner wells and allows well-to-well communication via the overlying medium. The test material or toxicant is added to the flooding/overlying media providing simultaneous and uniform distribution to all cell types. After incubation of the test material (typically 24-48 h), the overlying medium can be analyzed for overall metabolism of the test material, and individual cell types can be processed for the quantification of associated test material and specific biologic responses such as proliferation, apoptosis, cell cycle arrest, gene expression, and cytokine production.

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and drink containers while epoxy resins are used as protective liners in metal cans. Hence, exposure to BPA is likely to occur by consumption of foods and beverages that have contacted these materials (Le et al., 2008). In this study, human hepatocytes, renal proximal tubule cells, and small airway epithelial cells were treated simultaneously with varying degrees of BPA. As shown in Figure 2, BPA was toxic to all three cell types at high concentrations of 200 µM. However, at lower concentrations of 22.2 and 66.7 µM selective toxicity towards hepatocytes was observed. Hepatotoxicity of BPA has been reported both in vivo and in vitro using microsomal activation systems and has been attributed to the formation of DNA adducts in the liver. This effect was attenuated by known inhibitors of CYP450 enzymes, indicating bioactivation by hepatic metabolizing enzymes (Atkinson and Roy, 1995). Other possible mechanisms of toxicity include generation of reactive oxygen species and oxidative stress (Kovacic, 2010), as well as pronounced effects on the estrogen receptor (Li, 2008; Kurosawa et al., 2002).

IdMOC, in addition to being a multi-organ model, can serve also as a single organ model (Kurosawa et al., 2002). Co-culture of three human pulmonary cell types was established using normal bronchial epithelial cells (NHBE), small airway epithelial cells (SAEC), and human microvascular endothelial cells (HMVEC), and it was treated with increasing doses of different cigarette smoke condensates. Flue-cured tobacco was found to be the most cytotoxic compared to commercial “light” and “full flavored” cigarettes, Burley tobacco, or commercial “filter”-containing cigarettes. These results were comparable to those observed in assays using human lymphoblastoid (TK-6), Balb-c3T3 or Chinese Hamster Ovary (CHO) cells. Increased proliferation, however, was detected at low concentrations of flue-cured tobacco and “light” cigarette blends only in the IdMOC model (Richter et al., 2010). In light of the fact that cigarette smoking has been associated with lung cancer (Wynder and Hoffmann, 1976), these results are physiologically relevant. It is also apparent from these studies that the use of multiple cell types in a single experiment increases the likelihood of detecting cell-type specific toxicity, and overcoming the limitation of choosing only one cell type for an experiment.

5.2 Detection of metabolic activation in IdMOC

IdMOC can be used to detect bioactivation of a parent compound to stable, diffusible, toxic metabolites by co-culturing a metabolically competent cell type (e.g., hepatocytes) with a metabolically incompetent culture (e.g., CHO cells; mouse 3T3 fibroblasts). Cyclophosphamide, a compound that requires metabolic activation to exert its cytotoxic effects, is found to cause toxicity to the metabolically incompetent cells only when co-cultured with hepatocytes (Li, 2008; Li et al., 2012). The studies confirm a major application of the IdMOC system, namely, the evaluation of the effects of hepatic metabolism on the toxicity of a xenobiotic towards nonhepatic cells.

Richter et al. incubated TK-6 cells with phenobarbital and β-naphthoflavone induced rat liver S-9 fractions for metabolic activation of cyclophosphamide and tested several smoke cell condensates in this assay. They found that not only cyclophosphamide but also flue-cured and burley tobacco, along with filter containing cigarette condensates, were biotransformed.
into toxic metabolites causing a significant decrease in viability (Richter et al., 2010). Another approach is the transfer of supernatant from a chemically treated metabolically competent cell onto the target (metabolically incompetent) cell (Bruinink and Maier, 2007). While this approach has been used successfully, dilution of the toxic metabolite may prevent detection of the biological response in some cases.

5.3 Pharmacokinetic analysis in IdMOC

Cultivation of multiple cell types in the form of a cellular array in IdMOC plates opens up the possibility of performing drug metabolism studies in a more physiologically relevant manner. Drug stability and metabolite formation can potentially be evaluated after metabolism by cells from multiple organs. Drug distribution also can be estimated in multiple cell types after single or repeated exposure to the xenobiotic. Evaluation of the pharmacological responses to the test material in cells from both the target and non-target organs can be used to predict drug efficacy and off-target effects (Li, 2007a).

Further technological advances such as availability of luminogenic substrates of CYP450 enzymes (Promega, Inc.) allows one to monitor enzyme activity in cells while siRNAs or dominant negative cDNAs can be used specifically to inhibit CYP pathways or other metabolizing enzymes and transporters within cells and, hence, validate their role in metabolism. Hepatocytes from commercially available knockout animals such as HRNTM or PXR mice can be used to study the role of P450 metabolism or the Pregnane X receptor, respectively, in drug metabolism. Targeted disruption of specific transporters such as MDR1a (Multi-Drug Resistance gene-1a), OCT1 and 2 (Organic Cation Transporter genes Slc22a1 and Slc22a2), and MRP1 (Multiple Drug Resistance Protein 1), also are useful in validating the contribution of such proteins in drug metabolism and excretion.

5.4 Limitations of IdMOC

One drawback of the IdMOC model is that there is no actual directed flow of the overlying medium from one organ-specific cell type to another. Hence, this system cannot model certain events such as delivery of a bolus dose to the liver or differential exposure due to organ-specific blood flow. With the advent of microfluidics in biological applications, however, one can envision a closed IdMOC system where the inner wells are connected by integrated capillaries and the flow of the interconnecting medium is controlled by a pressure-driven pump. Potentially, such a development could mimic vascular blood flow and enable high-throughput in vitro screening alternatives for the study of xenobiotic metabolism and toxicity.

6 Conclusions

In keeping with the evolving regulatory needs of testing thousands of chemicals in a short period of time, innovative and high throughput assays for chemical testing are being developed. Replacement of animal testing with in vitro cellular assays, along with the use of intact human cells, has been recommended. Incorporation of metabolism into in vitro testing assays, as well as the use of multi-cellular models, have been largely lacking in the development of this new generation of toxicity assays. Metabolism is a key aspect of chemical toxicity. A chemical can be activated from a non-toxic parent molecule to toxic metabolites or, conversely, can be detoxified via biotransformation into non-toxic metabolites. While metabolite identification studies carried out with hepatocytes or microsomes can identify potentially toxic metabolites, subsequent studies on cellular models are required to evaluate toxicity. The presence of target cells with metabolically competent cells, as observed in the IdMOC system, would provide the means to incorporate metabolism into a toxicity assay. Such a model would be more physiologically relevant than current standards. The cultivation of multiple cell types in IdMOC plates without physical mixing allows evaluation of multiple endpoints such as proliferation, apoptosis, gene expression, and enzyme activity. Moreover, several pharmacokinetic studies such as metabolic stability, profiling, drug distribution, and evaluation of drug-drug interactions, can be studied in the presence of multiple cell types. The IdMOC model mimics interactions between different cell types, either within an organ or between multiple organs, thus providing a physiologically relevant model for evaluation of chemical toxicity.

References


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3D In Vitro Living Systems for Biological Application

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Summary
Many three dimensional (3D) models currently in practice require expensive equipment, large sample volumes, long incubation times and/or extensive expertise. Their most serious disadvantage, however, is that they are too far from the nature of human organs. Because of the above problems, research and development in drug discovery, regenerative medicine, biotech, and pharmaceutical industries is very costly, and it takes several years to bring a single drug/product to the market. The goal of our research is to merge biomaterials science, nanotechnology, and biological principles to generate 3D in vitro living organs, to be called “Human on a Chip.” The goal is to mimic organs/tissues in order to partially reduce the amount of in vitro testing, in vivo animal testing, and clinical trials, as well as to solve the problems noted above. In short, our goal is to jump “from lab bench to the market.” We propose to do all the above costly and time-consuming tests in a rapid and cost-affordable way. At the nanoscale, we play with the chemistry and materials to fabricate novel types of hydrogels that are similar to human organs, infusing the cell-laden hydrogels with extracellular matrix (ECM) molecules and gradients of signaling molecules to influence cell development and aggregation. On the microscale level, we employ fabrication technologies such as photolithography, adopted from the semiconductor industry, to mass-produce identical building blocks in a variety of shapes and sizes. These products will have to mimic the physical, chemical, and biological properties of natural organs and tissues at different scales, from molecules to cells to building blocks to organized clusters, if we are to reach our target, i.e., “Human on a Chip”. It is envisioned that the proposed method can be used to generate vascularized organs and tissues with controlled cell-cell and cell-matrix interactions that will be useful as in vitro diagnostics tools, for drug screening applications, and for transplantation.

Keywords: HTS, biosensors, 3D in vitro systems, human on a chip, microscale technology

1 Introduction
In the past few years, many high-throughput screening (HTS) technologies together with significant efforts to develop diagnostics tools such as biosensors have revolutionized the process of drug discovery to screen hundreds of thousands of compounds against disease targets to find novel drugs. Typically HTS studies are performed through a combination of modern robotics, data processing and control software, liquid handling devices, and sensitive detectors. However, robots and other equipment required for HTS studies are often expensive. Large volumes of cost-intensive chemicals are required to perform such tests. Another limitation is the time it takes to assay individual samples. In the end, even as these sets of facilities are fixed, the experimental data are not reliable for clinical application and, therefore, animal testing and clinical trials are required in order to get approval for the product (drug) after a very long processing time. The above problems are among the many limitations of current technologies using materials in many aspects of biological applications. Despite considerable effort at developing methods to enable testing of many conditions, it still takes a highly specialized and expensive screening lab to run a HTS operation, and these studies often are limited to large or moderately sized research institutions. The purpose of our study is to use microengineering, biomaterial sciences, and biological principles to develop 3D in vitro living organs with controlled microvasculature and tissue architecture. These 3D models will be useful for in vitro diagnostics and drug screening applications as well as for transplantation. Furthermore, 3D in vitro living organs contribute significantly to the development of microscale biosensors that will be more portable and scalable for point-of-care sample analysis and real time diagnosis. Modern biosensors based on micro- and nanoscale techniques have the potential to greatly enhance methods of detecting foreign and potentially dangerous toxins and may result in cheaper, faster, and easier to use analytical tools.

Microscale technologies are potentially powerful tools for addressing some of the challenges in 3D in vitro models. MicroElectroMechanical Systems (MEMS), which are an extension of techniques used in the semiconductor and microelectronics industries, can be used to control features at length scales <1 μm to > 1 cm³. In the past few years, microfabrication has been used increasingly in biomedical and biological applications. This is partially due to the emergence of such techniques as soft lithography to fabricate microscale
devices without the use of expensive “clean rooms” and photolithographic equipment. These techniques are compatible with cells and are now being integrated with biomaterials to facilitate the fabrication of cell-material composites that can be used for biomedical engineering (Lindstrom et al., 2010). In addition, microscale technologies provide an unprecedented ability to control the cellular microenvironment in culture and to miniaturize assays for high-throughput applications. Initial experiments used micromachining technologies on silicon surfaces to generate vascularized systems. Subsequent work on the replica molding of biocompatible polymers such as poly(dimethylsiloxane) (PDMS) from patterned silicon wafers has resulted in the fabrication of biocompatible scaffolds. More recently, microfabricated capillary networks have been fabricated out of biodegradable elastomers such as poly(DL-lactide-co-glycolide) (PLGA), polyurethane, and poly(glycerol sebacate) (PGS). However, there are potential disadvantages with the use of these polymers, including their rigid mechanical properties and bulk degradation kinetics. Alternative methods of fabricating scaffolds with micro- and nanoscale resolution include 3D printing, microsyringe deposition, tissue spin casting, and electrospinning of nanofibers. These approaches, however, are difficult to perform and to scale up for fabrication of large 3D organs. A potentially powerful approach to engineering the microvasculature is to use cell-laden hydrogels. Recently, it has been demonstrated that biomaterials made from hydrogels can be molded to fabricate microchannels. Although this technology has not yet been used for 3D living models, the ability of this approach to mimic organs and tissues is useful in engineering complex, vascularized organs.

One of the main features of the proposed approach is that it will use micro-engineered cell-laden hydrogels for 3D in vitro living organs. These hydrogels mimic the natural tissues in that they provide a 3D environment for cells. To use hydrogels in various biological applications, it is desirable to control their mechanical properties which affect cell attachment, differentiation, viability, and proliferation. Therefore, hydrogels that can mimic the mechanical, biological, and physical properties of native tissues need to be generated. Despite significant progress, however, many current approaches to fabricating hydrogels fail to result in the synthesis of constructs with the desired mechanical and chemical properties. Limitations in generating robust hydrogels that can withstand the in vitro environment include the need for low overall concentration of material, the required degradation, and the need for cytocompatibility. Our research aims to use interpenetrating networks (IPNs) as a powerful method of modifying hydrogel properties. Furthermore, we will be able to tailor the mechanical, physical, chemical, and biological properties of these hydrogels. Over the years, much has been attempted in generating tissue-engineered products. One strategy for engineering 3D engineered tissues is to cultivate cells within biodegradable scaffolds made from either natural or synthetic materials. A major challenge in 3D tissue engineering is that cells quickly lose their differentiated function. This is in contrast to the behavior of cells in the body, which have the capacity to regenerate. Thus, it is desirable to formulate alternative approaches to more precisely control the organization of cells and the vascularization of engineered tissues. Traditional 3D scaffolding approaches are not suitable for generating such complex structures due to lack of control of the tissue architecture and cell-cell interactions. In particular, cells in 2D culture, as well as within traditional 3D scaffolds, simply do not organize as they do in normal tissue; their metabolic properties are, therefore, unsuitable for tissue engineering applications. Our research plan aims to make an advance in 3D models by developing the basis for fabricating tissues made from cell-laden hydrogels with engineered microvasculature. Although engineering microscale features into tissue engineering scaffolds has been attempted before, in this proposal we will use a cell-laden hydrogel, which will eliminate the difficulties associated with other microfabricated tissue engineering scaffolds, such as uniform cell-seeding.

2 3D in vitro technology

Materials technology aims to develop a technique that draws from microscale engineering, novel biomaterials, and biological principles to overcome the limitations of the current approaches to generating 3D in vitro living organs. These include the inability to generate 3D constructs that mimic the complexity of native tissue structure, or to generate vascularized structures within a 3D tissue culture system. The success of hydrogels as tissue implants or biomedical devices is strongly dependent on their bulk properties. The ability to seed cells directly within macroporous hydrogels will be important for overcoming challenges associated with uniform cell seeding density and vascularization. However, most hydrogels lack the desired mechanical and biological properties associated with human tissues in the body. IPN composed of different types of natural hydrogels is a very attractive technology for overcoming the above problem. We used three different hydrogels in our previous studies (Hosseinkhani et al., 2006a,b,c, 2007a,b,c, 2010; Tian et al., 2008; Mohajeri et al., 2010). Mixtures of collagen/fibronectin, photocrosslinkable HA and self-assembled PA are used to synthesize IPNs (Fig. 1) comprised of the various combinations of the three different hydrogel precursors to generate a library of IPN hydrogels.

An example is the fabrication of five different IPN of collagen and HA at the following mass ratios: i) 20% collagen, 80% HA, ii) 40% collagen, 60% HA, iii) 50% collagen, 50% HA, iv) 60% collagen, 40% HA, and v) 80% collagen, 20% HA. These concentrations allow us to measure the mechanical properties of the entire spectrum of each binary IPN. Finally, IPN consisting of all three of the precursor solutions at 20 different concentrations again allow us to study the properties of the entire spectrum of IPN. Based on testing at least five different concentrations for each of the three binary IPN conditions (i.e., IPN of HA-PA, HA-collagen and collagen-PA), as well as the 20 different concentrations of triple IPN (i.e., HA-PA-collagen (Fig. 2), the total number of experiments can easily be performed using standard laboratory
techniques. With these approaches it is possible to synthesize and test biological and mechanical properties of the library of hundreds of different polymers by using liquid-handling robots, thus allowing us to easily screen for thousands of polymer combinations and concentrations. The polymers are synthesized on the arrays in triplicates to ensure statistical validation. We will synthesize a library of IPN hydrogels and analyze their mechanical properties by a high-throughput nanoindentor technique. The nanoindentor approach can be used to accurately and rapidly assess the load-displacement responses of the various polymers within the library. Briefly, the arrays are printed in triplicates using a microarrayer (MicroGrid TAS) on a standard PDMS substrate that is fitted with small microwells to ensure proper positioning of the individual hydrogels. These wells act as a placeholder and will not affect the unconfined compression testing since they will not be deep relative to the height of the hydrogels. With this approach we are able to design a hydrogel that can mimic mechanical properties of human organs and tissues. In general, brain tissues exhibit elasticity between 0.1kPa and 1kPa, muscle tissue ~10kPa, and collagenous bone approximately 100kPa. The human heart has an elasticity of ~31 kPa.

Fig. 1: Schematic diagram of interpenetrating networks

Fig. 2: Ternary diagram of the various IPN compositions that will be investigated in this study
Proposed samples include the 3 individual hydrogel precursors, 14 binary IPN combinations, and 20 ternary IPN mixtures.
will be duplicated and continued to culture the 3rd and the 4th and more layers of the cells, by which we are able to create in vitro living organs to be used as intelligent diagnostic tools.

To generate micropatterned arrays of libraries on IPN hydrogel and to enable their testing in a high-throughput manner without the use of expensive equipment or large reagent volumes, these hydrogel arrays can be manufactured at a central facility, dried and stored until testing. The ability to fabricate the microarrays at a central location eliminates the need for the end users to employ robotics or other types of equipment. To test each sample, the microgel array is simply sealed against an array of microwells containing cells in culture media. Upon exposure to the aqueous environment the chemical is released. By this approach, we are able to develop the individual components for this system, including an array of chemical-containing hydrogels immobilized on a hydrogel slide and an array of microwells, as shown in Figure 3.

**Fig. 3: Schematic representation of the in vitro based biochip**
A detection system for released proteins from cells in the microwells. Antibodies are printed on the array and binding of the proteins to the antibodies can then be detected by ELISA and a microarray scanner.

### 3 Fabrication technologies for 3D models

Fabrication technologies such as photolithography are borrowed from the semiconductor industry to mass-produce identical building blocks in a variety of shapes and sizes. For fabrication of 3D in vitro living systems we use an advanced lab of microfabrication facilities based on lithography technology through collaboration with the semiconductor industry toward the future development of biochip technology. The use of microfluidic channels or micromolding techniques to deposit cells and materials on specific regions of a substrate that may also be used for microfabrication is important as well. To engineer 3D living organ models, soft and photo-lithography techniques are followed. In this approach, the first layer of cells is cultured in IPN hydrogels; following photolithography, a cell-laden microwell will be formed. The next step is culturing the 2nd layer of the cells inside the empty microwell. This process will be duplicated and continued to culture the 3rd and the 4th and more layers of the cells, by which we are able to create in vitro living organs to be used as intelligent diagnostic tools.

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By incorporating the engineered organs inside arrayed microfluidic channels, we can fabricate the tissue-based diagnostic tools for a drug screening system, for instance. A technique required by these proposed experiments is to pattern an array of hydrogels containing different drugs on a PDMS substrate. A commercially available spotter (MicroGrid II TAS) is utilized to selectively immobilize desired combinations of matrix molecules within each microwell, as well as a second microarrayer (PerkinElmer) that utilizes piezoactuation to print droplets of liquids on substrates. The fabrication process of hydrogel arrays requires access to a standard robotic spotter, off-the-shelf materials, and 1,000 times less reagents than conventional methods of testing molecules on chemicals (Fig. 1). Approximately 3,000 unique spots are patterned in less than 30 minutes, making the process amenable to high-throughput screening. Furthermore, microarray printers can be used to print high-molecular-weight PEG molecules that can be used to fabricate our proposed arrays.

Two kinds of concepts will be applied here. First, the microfluidic channel in which the 3D-vascularized model is placed later carries out the chemical assay by flowing different chemicals through each channel. Second, the 3D vascularized models inside the gradient-generating chip are fabricated. By flowing the drug (or chemical) having a gradient through the channel, the 3D model is exposed to the chemicals at different concentrations and, therefore, we are able to investigate the chemical concentration effect on organs or tissues. The microfluidic platform containing multiple channels is fabricated using PDMS, and the flow networks having different reserves are connected to each chamber. The width of each channel is less than 1 mm. The microfluidic platforms are combined to fabricate the arrayed assay chip. Then the cell-laden hydrogel is introduced through the channel and is polymerized. The system including 3 kinds of cells is incubated, and we can fabricate the arrayed channel with the 3D engineered model inside. Finally, we can analyze the drug (chemical) effect on specific tissues by flowing different chemicals through the microfluidic channels.

The 3D engineered vascularized model is placed inside the gradient-generation chip. The proposed gradient-generation chip is simple and requires only a small amount of chemical because the flow is driven by osmotic pump. This chip can easily be used inside the cell incubator for a long time (>48 h) without changing media. The media or chemicals can be easily supplied, and the cells can be incubated for much longer times (i.e., more than 1 week). We put the media without chemicals in one reservoir and the media with chemicals in the other reservoir. Then the chemical gradient is generated inside the channel, and the 3D vascularized organs or tissues are influenced by the gradient of chemicals. By monitoring the tissues exposed to different drugs or chemical gradients, we can investigate chemical gradient effect on cells, and this concept can be applied to the high throughput screening of tissues according to different chemicals and concentrations.

This technique offers great flexibility in testing drug or chemical libraries. For example, various doses of the same chemical can easily be tested from the hydrogel microarrays to assess the minimum toxic dosage. This can be used in arriving at toxic dose levels without expensive and ethically challenging animal experimentation. In addition, the system can be modified to conduct other high-throughput experimentation. For example, various differentiation factors at different concentrations can be printed in the hydrogel slide, and their effects on the cell’s behavior can be studied in a multiplexed manner.

4 Milestones, deliverables, and economic potential

We anticipate that elucidation of the above goals will open many doors and lead to significant improvements in biological tools and the drug discovery process, as well as to identification and therapeutic approaches. The miniaturization of this approach allows one to perform many more experiments and to do so more simply than previously possible.

The 3D in vitro technology aims to develop a set of tools that are simple, inexpensive, portable, and robust and which could be commercialized and used in various fields of biomedical sciences, such as drug discovery, diagnostic tools, and therapeutic approaches in regenerative medicine. Our research program will be interdisciplinary, building the critical and experimental research media with the aim of overcoming the fundamental limits to information processing. It will enhance graduate education in the local universities, encouraging high quality researchers among local students to pursue research careers and create new knowledge in selected areas of focus. Its top academic and high-tech objective is to fabricate, investigate, and implement novel advanced micro- and nano-structures and superstructures based on ordered, highly functionalized materials to meet the demands of maximum efficient, active hydrogel materials of high and sustained reactivity as well as long term stability. It will enable new fundamental research and development for the next generation of biomedical materials, as well as the exploitation of such novel structures in the development of novel biomedical devices, transferring the knowledge to academia and industry for future implementation of novel knowledge and technology to the world.

In turn, it should increase the international competitiveness of the world in knowledge-intensive micro- and bioengineering of organ-based biosensors by focusing on high-impact research to generate new breakthroughs aimed at solving significant practical problems of biomedical sciences while seeking to extend the boundaries of understanding. It aims to raise the research profile of bioengineering as a vibrant center for medical and technological applications through a bottom-up approach that embraces elements of both basic and applied research to enhance the competencies in existing technologies and to seek out promising new areas and to develop an integrated, cutting edge research program by growing a pool of top research talent and developing the platforms on which local universities could create research breakthroughs of importance to the world.

Since advancement in device development technologies is a significant indicator of developed societies, the rapidly growing market for biomedical devices provides a competitive
advantage in R&D and commercialization for this field. Such advancement can be reached by helping local universities to set up and establish their own facilities using our technology created in local universities.

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Testing Times in Toxicology – In Vitro vs In Vivo Testing

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Summary

Initiatives such as the “Human Toxome Project,” which aims to map the “Pathways of Toxicity” (PoT) in man illustrate a trend that moves away from our current reliance on high-dose animal toxicity studies to a wide range of new tools such as functional genomics, proteomics, metabolomics, high data content screening, pharmacokinetic modeling, and systems biology to study the effects of chemicals on cells, tissues, and organisms in a rapid and cost-efficient manner. These technologies are also paving the way to improve the evaluation of health risks posed by chemicals found at low levels in the environment. This convergence of factors, coupled with the need to evaluate the safety of an increasingly large number of chemicals and their mixtures, has prompted a call for a fundamental paradigm shift in toxicology testing.

In this scenario, we are seeing a sure and fast transition in toxicology testing from animal tests to in vitro methods, virtual tissues, robotic automation, and beyond, where mathematical modeling, structure activity relationships of chemical molecules, and computational toxicology are being used to predict toxicity with precision and high throughput.

This brings us back to the eternal philosophy that says the ultimate search for truth culminates in nonviolence. As Indians we cannot but honor the Great Mahatma, who said, “I abhor vivisection with my whole soul. All the scientific discoveries stained with innocent blood I count as of no consequence.” The science of alternatives is to know that humane science is better science, being precise, predictive, and pain-free.

Keywords: toxicity testing, in vitro, in vivo, animal alternatives

1 Introduction

“Absence of evidence is no evidence of absence” – Carl Sagan, US astronomer (1934-1996)

In the context of animal toxicity testing Hartung and McBride (2011) evoked the above quote to explain the fallacies we see in live animal testing. However, for reasons unknown or maybe due to the lack of better and more humane testing strategies, animal testing was in the past and, unfortunately, continues to be the gold-standard in the toxicity evaluation of pharmaceuticals, cosmetics, agrochemicals, and industrial chemicals. Even with the evolution of science to more robust, precise alternatives, both “humane and human,” the shift has been slow despite the fact that regulatory authorities, both in the USA and the EU, have withdrawn close to 50 important drugs from the market in the last 50 years, even after they had been proved safe/efficacious, having been tested with the whole gamut of animal studies and clinical trials as prescribed by regulatory agencies. The worst and biggest tragedies resulted from the use of thalidomide and sulfanilamide. A more recent drug (drotrecogin alfa), once believed to be useful in treating patients with severe sepsis, has been withdrawn from the market because new evidence revealed – 10 years after the drug was approved by the US Food and Drug Administration (FDA) – that it is not effective for severe sepsis after all. This drug was withdrawn by Eli Lilly and Company on October 25, 2011.

False negatives have been one of the biggest and seemingly insurmountable problems in animal testing. A negative animal test could mean nothing or so many things. A different species or simply a different experimental variation may yield positive results. Animals have different defense mechanisms that may lead to false or skewed results, and the stress of being caged, being held in unnatural conditions, and being handled, in itself, could be responsible for result vagaries. Furthermore, the rodent to human extrapolation factor, which is intrinsic and inevitable to animal toxicity data, must be considered. Physiological responses to stress may be an add-on to a positive result, camouflage a toxicity indicator, pose variations to a toxicity pathway, exaggerate, or maybe just trigger a toxicity pathway that may be absent in the absence of stress or another variable.

The scientific understanding of how genes, proteins, and small molecules interact to form molecular pathways that maintain cell function has evolved rapidly, thanks to advances in molecular and computational tools. This knowledge gives non-animals methods the edge over in vivo testing, taking away that adjunct of variations that come in animal testing due to the pain, stress, and distress that laboratory animals suffer during
an experiment. This unassessed and un-quantified variable of stress interferes with physiological processes and disrupts critical pathways, interfering with the experimental design, results, and observations, thereby underplaying or exaggerating the ultimate inference of the level of toxicity. The absence of this variable in non-animal testing makes the tests more robust, adding high throughput and high precision, rendering a better prediction potential. Reconstructed human tissues, “neurons on microchips,” “organs-on-a-chip,” and “brains-in-a-test tube,” computational toxicology, and “-omics” have taken toxicology testing to greater heights in human risk assessment, allowing scientists to work with real-world human exposure levels and real-life predictable, human results.

2. The evolution of testing strategies in pyrogen testing – An example of the development of a “humane and human” alternative

Twenty years ago, if one spoke of “fever in a test tube,” it would have been seen as the proverbial rabbit-from-the-black-top-hat kind of magic, rather than a scientific discovery. In the late 1990s – somewhere between the rabbit and the test tube in the history of pyrogen testing – came the Horseshoe Crab Assay (Limulus Amoeocyte Lysate Assay or LAL), which was an effective replacement for the rabbit pyrogen test. It was based, however, on the hemolymph of the horseshoe crab. In 2009, the evolution of a non-animal alternative in pyrogen testing took another step forward in replacing the ubiquitous rabbit pyrogen test. This time, the world saw a ground-breaking discovery with the use of cryopreserved human whole blood as an effective and efficient tool for detecting fever-causing bacteria. No rabbits – with or without a magic hat – are used in this test.

In this in vitro pyrogen test, where one tests fever in a test tube, the sample is incubated with fresh or cryopreserved human whole blood, and the proinflammatory cytokine interleukin-1β (IL-1β) is detected by an enzyme-linked immunosorbent assay (ELISA). In addition to detecting pyrogenic contaminations in aqueous samples, e.g., parenteral drugs, adaptations allow the assessment of lipidic, toxic, or immuno-modulatory substances; detection of low-grade contaminations in large-volume parenterals, e.g., dialysis, water, and fluids; pyrogenicity assessment of solid materials, e.g., medical devices; and evaluation of airborne pyrogenic burden. It is superior to both the rabbit pyrogen test and the Limulus Amoeocyte Lysate (LAL) test in that it also detects nonlipopolysaccharide pyrogens, and the procedure takes 21-35 h to complete (Daneshian et al., 2009).

3. Toxicity testing and the use of animal alternatives – The 21st century scenario

3.1 The ToxCast program

The United States Environmental Protection Agency (EPA) launched ToxCast\(^1\) in 2007 to develop ways to predict potential toxicity and to develop a cost-effective approach for prioritizing the thousands of chemicals that need toxicity testing. ToxCast uses advanced science tools to help understand how human body processes are affected by exposure to chemicals, and it helps determine which exposures are most likely to lead to adverse health effects. ToxCast testing methods include more than 650 state-of-the-art rapid tests (called high-throughput assays) that can screen more than 2,000 environmental chemicals for potential toxicity. Phase I, “Proof of Concept,” was completed in 2009, and it profiled more than 300 well studied chemicals (primarily pesticides). Phase I chemicals have more than 30 years’ worth of existing toxicity data, since they have been tested already using traditional toxicology methods (primarily animal studies). Data from animal studies can be searched and queried using EPA’s Toxicity Reference Database (ToxRefDB) that stores nearly $2 billion worth of studies. Phase II currently is screening 1,000 chemicals from a wide range of sources, including industrial and consumer products, food additives, and drugs that never made it to the market, to evaluate the predictive toxicity signatures developed in Phase I. Data from the high-throughput assays is available via the ToxCast Database. Toxicity signatures from ToxCast are defined and evaluated by how well they predict outcomes from mammalian toxicity tests and identify toxicity pathways relevant to human health effects.

3.2 The Tox21 program

The robotic testing of a 10,000-compound library marks the beginning of a new phase of toxicity testing. An ongoing venture of the National Institutes of Health, the U.S. Environmental Protection Agency, and the U.S. Food and Drug Administration, referred to as Tox21\(^2\), was launched on December 15, 2011. It signifies a collaboration that will move science forward. In this program 10,000 chemicals will be screened for potential toxicity by a high-speed robotic screening system. The project aims at protecting human health by improving the way chemicals are tested in the United States. The compounds cover a wide variety of classifications and include consumer products, food additives, chemicals found in industrial processes, and human and veterinary drugs. Each test compound will undergo a thorough chemical analysis to verify its identity and determine its purity, concentration, and stability. The goal of the testing is to provide results that will be useful for evaluating whether these chemicals have the potential to disrupt processes in the human body to an extent that leads to adverse health effects. The Tox21 partnership integrates advances in molecular biology, chemistry, and computer science, enabling fast and low-cost

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2. [http://www.epa.gov/nct/Tox21/](http://www.epa.gov/nct/Tox21/)
screening of the thousands of chemicals in use, and it will help generate the toxicity data of these chemicals, which in turn will enable the production of safer drugs and also help identify unsafe drugs.

### 3.3 The Human Toxome project

Taking toxicity testing to a still higher platform are scientists who have embarked on a pioneering and novel project along the lines of the “Human Genome Project,” in this case called the “Human Toxome” project, led by Dr Thomas Hartung, Director, CAAT, Johns Hopkins University. The project envisages the mapping of the “Pathways of Toxicity” (PoT) in man. PoT include pathways of endocrine disruption, perturbations of the hormonal system or other physiological disruptions that manifest themselves as adverse health effects, tumors, birth defects, developmental disorders, etc. The vision is to produce a suite of in vitro, sub-cellular, and in silico tools that comprehensively represent the human toxome. Once a PoT is identified, the construction of the test system will not be difficult (Hartung and McBride, 2011). Currently, toxicity testing typically involves studying adverse health outcomes in animals subjected to high doses of toxicants, with subsequent extrapolation to expected human responses at lower doses. At present, humans are potentially exposed to more than 80,000 chemicals for which no toxicity data exists.

According to the same authors, the challenge we face as scientists is to turn around the testing paradigm of regulatory safety assessments from phenotypical tests to tests based on a mechanistic understanding identified on the basis of known human toxicants. According to them, our current understanding of systems biology and a host of molecular, informational, and computational tools provide the potential to identify PoT to evaluate the effects of tens of thousands of chemicals at concentrations relevant to human exposure levels. “In contrast to the currently used phenomenological “black box” animal testing, pathways of toxicity (PoT) will be identified in human in vitro systems to provide more relevant, accurate, and mechanistic information for the assessment of human toxicological risk,” they opine. The goal is to map the entirety of the human toxome. The concentration at which a substance triggers a PoT then can be extrapolated to a relevant human blood or tissue concentration and, finally, a corresponding dose by (retro-) PBPK (physiology-based pharmacokinetic) modeling, thereby informing human risk assessment. Perhaps more importantly, if a substance does not trigger any PoT, it may be possible, for the first time, to establish the lack of toxicity, i.e., safety of a substance at a given concentration. A comprehensive list of PoT, the mapped human toxome, can become a cornerstone of this new regulatory toxicology. Project “Human Toxome” will use integrated testing strategies that combine transcriptomics and metabolomics data with computational models with a view to creating a public database of PoT, enabling full access to researchers around the world. This understanding of toxicity pathways could serve as the background, rendering the basic cues for a humane or, more importantly, a “human” alternative in toxicity testing.

### 3.4 AXLR8

Advances made in molecular biology, biotechnology, and other fields are paving the way for major improvements in how scientists evaluate the health risks posed by potentially toxic chemicals found at low levels in the environment. These advances would make toxicity testing quicker, less expensive, and more directly relevant to human exposures. They also could reduce the need for animal testing by substituting more laboratory tests based on human cells.

Based on this premise, the AXLR8 project has been launched by the European Union. The European Commission currently is funding a number of research consortia to develop new 3Rs (replacement, reduction, and refinement) test methods and strategies as potential alternatives to the use of animals in safety testing. AXLR8 aims to fulfill this growing need by providing a focal point for dialogue and collaboration. Specifically, AXLR8 aims to:

- Organize a series of annual workshops to monitor research progress on alternative testing strategies.
- Provide a range of tools and opportunities for enhanced interdisciplinary and international communication, coordination, and collaboration in order to maximize the impact of available resources.
- Work to streamline regulatory acceptance procedures to provide for the uptake of validated 3Rs methods, including a smooth transition to 21st century systems as they become available.

The ultimate goals are to assess safety of a much larger number of substances and mixtures than is currently possible, more rapidly, efficiently, and cost-effectively than at present, and in systems that may be more relevant to toxicity in humans and that will be capable of identifying the cellular mechanisms at the root of toxicity and disease, using fewer animals and with the final aim of complete replacement of animals. Instead of focusing on signs of gross toxicity at high doses in living animals, AXLR8 will focus on the “21st century” approach advocated by leading scientific and regulatory authorities, working towards a mechanistic understanding of how chemicals interact with cellular response pathways in the human body at environmentally relevant exposure levels. As critical pathways are identified, human cell-based tests will be developed to study chemical interactions at key cellular and molecular targets within a pathway. Through robotic automation, cell-based in vitro methods will enable the high throughput testing of thousands of substances in a single day. Data from toxicity pathway assays then could be integrated and interpreted with the aid of systems biology tools controlling pathway function and be combined with pharmacokinetic modeling to relate in vitro conditions to real-world exposure levels.

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3 http://axlr8.eu/overview/
3.5 Sens-it-iv Project

Twenty-eight groups, of which nine represent industry, fifteen represent universities or research institutes, and four represent organizations in the EU, have joined hands to initiate the program Sens-it-iv\(^4\). The ultimate goal of this project is to develop an in vitro test strategy to predict the sensitizing potency of compounds, enabling the full replacement of animals in safety testing.

To date, there are no validated in vitro models for skin and respiratory sensitization. In addition, there is no widely accepted in vitro screening approach for early identification of potential sensitizers. Given the complex mechanisms involved in sensitization, it is unlikely that a single in vitro test will be able to substitute for the existing animal tests. Sens-it-iv attempts to overcome these limitations by exploring innovative approaches and integrating existing knowledge on the cellular and molecular mechanisms involved in sensitization based on assay systems that model sensitization, rather than irritation and toxicity of chemicals and proteins.

Moreover, since the cell culture models that will be used in Sens-it-iv are of human origin, they might prove to be more predictive than the current animal models of toxicity in man. This new knowledge also will help in designing safer drugs, vaccines, and many other materials of value to the EU.

Deliverables from Sens-it-iv will be in vitro tests that will be ready for formal validation according to international standards for subsequent international regulatory acceptance and, finally, for world-wide application in industry, regulatory establishments, and elsewhere.

3.6 Non-animal toxicity tests scientifically validated as alternative testing strategies for regulatory testing and biomedical research

Validation, in the context of non-animal toxicity testing, is the process by which the scientific prowess of a non-animal testing strategy is evaluated for the purpose of regulatory testing of agrochemicals, industrial chemicals, cosmetics, and pharmaceuticals. Validation criteria for new toxicological test methods in use today were developed by three organizations: the Organization for Economic Cooperation and Development (OECD), the European Centre for the Validation of Alternative Methods (ECVAM), and the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM). These organizations are sometimes called validation authorities.

More than 25 years ago, the OECD recognized the need to protect animals in general and, in particular, those used in experimental work. The progress in OECD on the harmonization of chemicals control, especially the agreement on Mutual Acceptance of Data (MAD), has helped greatly to reduce the number of animals used in testing by reducing duplicative testing. MAD states that data generated in the testing of chemicals in an OECD member country in accordance with OECD Test Guidelines and OECD Principles of Good Laboratory Practice (GLP) shall be accepted in other member and adhering non-member countries. These proactive Council Decisions still save thousands of animals every year, and an increasing number of non-OECD economies adhere to MAD.

The OECD is committed to implementation of the 3Rs principles (Replacement, Reduction, and Refinement), as first laid down by Russell and Burch (1959). Probably the most noteworthy achievement is the deletion of the much criticized Test Guideline 401 on Acute Toxicity Testing and its replacement with Test Guidelines 420, 423, and 425, introducing reduction and refinement. Other examples include the Local Lymph Node Assay (Test Guideline 429) introducing refinement and reduction compared to Test Guideline 406, and the Test Guideline 428 on “Skin Absorption: In Vitro Method,” offering an alternative method to Test Guideline 427, among others.

Another example of the commitment of the OECD to the implementation of the 3Rs principles into regulatory toxicity testing is the development of the Guidance Document No. 19 on “the Recognition, Assessment and Use of Clinical Signs as Humane Endpoints for Experimental Animals Used in Safety Evaluations” from 2000. Guidance Document No. 19 gives practical guidance on how to apply the 3Rs principles, with emphasis on refinements when performing OECD Test Guidelines.

The validated alternatives that can be used in regulatory and biomedical research are elaborated in Table 1. The INVITTOX database provides tried and tested in vitro toxicity assays that can be used in biomedical research. The aim of INVITTOX protocols is to present precise and up-to-date technical information on the performance of the in vitro techniques currently in use and under development, their applications, advantages, and drawbacks. The information is obtained directly from those scientists already employing such methods. Each protocol includes a detailed methodology sufficient to enable another researcher to carry out a procedure; experimental data, where available; the rationale for choice of technique and endpoint; and critical assessment comments about the accuracy of the system, its sensitivity, ease of implementation, shortcomings, etc. It is envisaged that making all this information available in one document will help scientists to select the systems most appropriate to their needs.

4 Ban on animal use in toxicity testing for cosmetics in the EU

An amendment to the European Union’s Cosmetics Directive (76/768/EEC) phases out the use of animals in testing for any acute toxic effects of beauty products and toiletries.

The Cosmetics Directive was introduced in 1976 to enforce high safety standards for cosmetics across the EU Member States. It was amended in 1993 to phase out the use of animals in testing, but the amendment was never implemented because

\[^4\]http://www.sens-it-iv.eu/
no alternative, animal-free tests had been approved. A stricter amendment developed in 2003 (Directive 2003/15/EC) forced a ban on the use of animals in the testing of finished cosmetics products within one year and imposed two further deadlines to phase out animal testing on any ingredient in a cosmetic product – regardless of whether alternative tests were available. As of March 11, 2009, the first of the two deadlines outlaws the use of animals in seven mandatory tests of toxicity following a single application. These are tests for skin irritancy, sensitivity to light, corrosivity, and absorption through the skin, genetic toxicity, eye irritancy, and acute toxicity. The amendment also bans the import of cosmetics containing ingredients that have been tested on animals in this way after the deadline.

The second deadline, March 11, 2013, would see a ban on eight tests designed to establish longer-term toxicity following multiple applications, for example their ability to cause cancer or birth defects. This deadline may be renegotiated, however.

Europe is a world leader in cosmetics, with global sales approaching € 80 billion (US $ 102 billion). That is nearly half of the world market. There are around 2,000 cosmetics manufacturers in the European Union, including some of the world’s largest, such as L’Oréal and Estée Lauder, and they sell five billion items every year.

Alternatives to four of the seven tests banned today have been validated and approved by the European Union, and work on the other three has advanced. During this time, the cosmetics industry will not be able to introduce new products that include chemicals not tested before the cut-off date. The European Union has been pumping € 35 million into efforts to develop alternative methods every year since the Cosmetics Directive was amended. EU Member States are estimated to put in a combined total of a further € 25 million per year. The European Union has implemented a systematic pan-European research program to find alternatives, coordinated with the smaller efforts that are going on elsewhere in the world (Abbott, 2009).

The cosmetics industry itself has contributed € 25 million for developing alternatives, which was matched by a further € 25 million by the European Union.

Tab. 1: Non-animal toxicity tests validated as alternative testing strategies for regulatory testing and bio medical research

<table>
<thead>
<tr>
<th>Test</th>
<th>Alternative (Replacement/Reduction/Refinement)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Eye corrosion</td>
<td>Bovine Corneal Opacity and Permeability (BCOP) assay and Isolated Chicken Eye (ICE) assay</td>
</tr>
<tr>
<td>Acute systemic toxicity</td>
<td>3T3 NRU assay / NHK NRU assay</td>
</tr>
<tr>
<td>Acute Oral Toxicity</td>
<td>Up-and-Down Procedure (UDP) / Fixed Dose Procedure (FDP) / Acute Toxic Class method (ATC)</td>
</tr>
<tr>
<td>Embryotoxicity/Teratogenicity</td>
<td>Embryonic stem cell test (EST) / Micromass test / Whole rat embryo culture</td>
</tr>
<tr>
<td>Phototoxicity</td>
<td>3T3 Neutral Red Uptake Phototoxicity Test</td>
</tr>
<tr>
<td>Skin corrosion</td>
<td>EpiSkin® – in vitro human skin model / Rat Skin Transcutaneous Electrical Resistance (TER / SkinEthic) – in vitro human skin model Corrositex® – noncellular membrane / Vitrolife-Skin RHE</td>
</tr>
<tr>
<td>Skin irritation</td>
<td>Reconstructed Human Epidermis Test – EpiSkin™ / EpiDerm™ SIT (EPI-200) / SkinEthic™ RHE</td>
</tr>
<tr>
<td>Pyrogenicity</td>
<td>Human cryopreserved whole blood IL-1 / Limulus Amebocyte Lysate (LAL) test</td>
</tr>
<tr>
<td>Skin sensitization</td>
<td>Local Lymph Node Assay: BrdU-ELISA</td>
</tr>
<tr>
<td>Reproductive toxicity</td>
<td>“Two-generation” animal test replaced by “extended one-generation” method</td>
</tr>
<tr>
<td>Endocrine disruptor screening assay</td>
<td>(ER)-alpha Transcriptional Activation Assay / H295R Steroidogenesis Assay / Aromatase Inhibition Assay / BG1Luc ER TA Test Method</td>
</tr>
<tr>
<td>Batch potency testing of vaccine</td>
<td>ELISA test for erysipelas vaccines / ELISA test for human tetanus vaccines / Toxin binding inhibition (ToBi) test for human tetanus vaccines</td>
</tr>
<tr>
<td>Monoclonal antibody production</td>
<td>In vitro MAb production systems</td>
</tr>
<tr>
<td>Genotoxicity testing</td>
<td>Mammalian Cell Micronucleus Test / Bacterial Reverse Mutation Test (Ames Test)* / Genetic Toxicology: Saccharomyces cerevisiae Gene Mutation Assay* / In vitro Mammalian Chromosome Aberration Test* / In vitro Mammalian Cell Gene Mutation Test* / In vitro Sister Chromatid Exchange Assay in Mammalian Cells* / DNA Damage and Repair, Unscheduled DNA Synthesis in Mammalian Cells In vitro*</td>
</tr>
</tbody>
</table>

*not validated but accepted by OECD/EPA
5 The Socio-scientific perspective to adopt alternatives and the Indian Scenario

“The thinking person must oppose all cruel customs no matter how deeply rooted in tradition or surrounded by a halo…. We need a boundless ethic which includes the animals also.” (Albert Schweitzer, Philosopher and Physician, Nobel Peace Prize Winner 1952)

Albert Einstein went on to say “Our task must be to free ourselves – by widening our circle of compassion to embrace all living creatures and the whole of nature and its beauty.”

Nonviolence has been an echoing and boundless truth, in all religions and in every philosophy, transcending every human culture, race, or profession.

The question posed here is: Today when we are in knowledge and convinced with evidence that alternatives to animal testing far excel in precision and prediction, does this not bring us back to history and the eternal philosophy that says the ultimate search for truth culminates in ‘Non-violence’? At this juncture, as Indians we cannot but honor Mahatma Gandhi, the “Father of our Nation,” who said more than half a century ago, “I abhor vivisection with my whole soul. All the scientific discoveries stained with innocent blood I count as of no consequence.” Every sage, saint, and philosopher spoke about “ahimsa” as the ultimate truth that goes beyond just being a way of life but that which has a bearing on every aspect of our lives, be it societal, environmental, or educational. “Ahimsa” is a truth that seems to go beyond scientific reason and mathematical algorithms.

India has surged ahead among nations in the last decade in promulgating laws favoring the welfare of animals and in promoting the science of alternatives (Shiranee, 2006). In the field of life science education, we have become a country to be emulated, with the University Grants Commission, Government of India, restructuring the Zoology curriculum with the ultimate aim of phasing out dissection (Akbarsha and Shiranee, 2010). Earlier, in 2004, the CPCSEA, the statutory body of the Government of India that controls and supervises the use of animals in experimentation, amended the Use of Laboratory Animals (Regulations & Guidelines) Rules, 1998, which mandates the use of non-animal methods to the extent possible, and allows the use of animals only as a last resort. This is in line with the Indian tradition of ahimsa and the Indian ethos of compassion for all living beings.

6 Conclusions

The underlying and essential point here is to comprehend that science is evolving, and evolving to better models, precision, and higher throughput, simultaneously giving us the benefit of establishing humane standards in toxicity testing. A welcome and undeniable need is that these developments are in recognition of the desire for better medical care and safety standards for humanity. Higher understanding of toxicokinetic studies will help in making safer and better life-saving drugs and will help set robust standards for better health care. Such global initiatives to move from our current reliance on high-dose animal toxicity studies to a wide range of new tools such as functional genomics, proteomics, metabolomics, high data content screening, pharmacokinetic modeling, and systems biology, to study the effects of chemicals on cells, tissues, and organisms in a rapid and cost-efficient manner, is the sweeping change we observe in toxicology testing today.

High-dose animal toxicity studies and the application of extrapolation procedures focus on signs of gross toxicity, which results in uncertainty when used in human health risk assessment, as observed by tragedies associated with new drugs/therapeutics, and dozens of drugs that have been taken off the market in the last century due to their adverse side effects in humans but that were not manifested or predicted by animal studies. The sooner we realize this need to change the better. It may be simply to realize, as Dr. Thomas Hartung succinctly observed, “We are not 70 kg rats.”

The scientific understanding of how genes, proteins, and small molecules interact to form molecular pathways that maintain cell function has evolved rapidly, thanks to advances in molecular and computational tools. This knowledge gives non-animals methods the edge over in vivo testing, taking away that adjunct of variations that arises in animal testing due to the pain and distress that laboratory animals suffer during an experiment. This unassessed and unquantified variable of stress, which interferes with physiological processes and disrupts critical pathways, interferes with the experimental design, results, and observations and, thereby, undermines the ultimate inference of the level of toxicity, essentially underplaying or exaggerating the toxicity. The absence of this variable in non-animal testing makes the tests more robust, adding high throughput, high precision, and a better prediction potential. Reconstructed human tissues, “neurons on microchips,” “organs-on-a-chip,” and moving further ahead to computational toxicology and “-omics,” have taken toxicology testing to greater heights in human risk assessment, giving scientists the possibility of working with real-world human exposure levels.

To promote this “humane science” of non-animal testing is to be able to understand that this is a science that needs to be promoted and practiced, not just for ethical reasons but in that it gives a three-fold advantage of being precise, predictive, and pain-free (the 3 Ps – Precision, Predictiveness, and Painlessness).

To quote William Russell and Rex Burch (1959): “If we are to use a criterion for choosing experiments to perform, the criterion of humanity is the best we could possibly invent. The greatest scientific achievements have always been the most humane and the most aesthetically attractive, conveying that
sense of beauty and elegance which is the essence of science at its most successful.”

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Reduction and Refinement Alternatives: Where, When, and How?

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Summary
“In vitro testing” is the paradigm of the current era, and it is gaining increased acceptance from various research sectors, including regulatory science. The impetus that this subject has gained is credited to the awareness and understanding that there is enormous scope and value associated with in vitro alternatives. First, alternatives can reduce a great many of the ethical issues associated with the use of animals in research. Second, there are numerous in vitro studies currently available that are potentially competent to reduce, if not completely replace, animal experiments. Considering the number of chemicals that are added every year and the amount of testing that goes into assessing each of these chemicals, it is worthwhile to encourage the use of in vitro alternatives that may be money-saving, time-saving, and certainly “animal-saving!” Thus, it is a moral responsibility to wake up to the situation and adopt existing, established in vitro methods for chemical testing, as well as to devise newer methods for potential use in the future.

Keywords: in vitro genotoxicity, reproductive toxicity testing, 4Rs, GLP

1 Introduction
Safety evaluation studies are regulatory requirements to fulfill a risk assessment mandate. Almost all new chemical, pharmaceutical, and biotech products entering the market should be assessed for intrinsic toxicity by a battery of in vivo animal studies covering acute to chronic and special studies such as reproduction, neurotoxicity, and others. Although extrapolation of animal data to man is not always possible or easy, these animal data could be used to avoid the introduction of a potentially toxic product. In the case of new drug development, in vivo animal data are used to identify target organs, if any, that form the basis for clinical trials. In the case of new chemical development, in vivo toxicity studies are used to make risk predictions by deriving non-observable effect levels (NOAEL), which in turn will be used to predict maximum residue limits (MRL), acceptable daily intake (ADI), and acute reference dose (ARD). Complex in vivo metabolism studies also were conducted in large lactating animals to find out the distribution and accumulation of the active compound and its metabolite in edible tissues/ fluid. Different countries have different guidelines and data requirements to register these products and efforts to harmonize these guidelines have met with only limited success. Consequently, there is often repetition of in vivo studies to meet the legal obligations in these countries.

2 Validated in vitro methods
Over the last 20 years, a number of validated in vitro methods have been developed as potential replacements for in vivo regulatory toxicity studies (Kandárová and Latašiová, 2011). The in vitro genotoxicity studies – micronucleus, chromosomal aberration, mouse lymphoma (gene mutation) studies, coupled with the classical Ames bacterial assay, are the most well-established battery of mutation studies, the results of which are widely used to draw conclusions on the mutagenicity of a chemical/drug/impurity.

3 Alternative methods that require fewer animals or no animals
New methods have totally avoided/replaced the painful and unethical in vivo studies in new chemical and drug development. The introduction of in vitro skin (OECD, 2004) and in vitro eye (OECD, 2009a,b) assays no longer require the use of rabbits. Use of artificial skin, biopsies, and bovine cornea from slaughterhouses are now used as alternatives for these tests. In addition, test guidelines have prescribed the conditions, such as pH, and previous information under which these studies can be limited, and therefore the animals’ pain during the test could be minimized. Reduction of the number of animals has been successfully achieved for LD50 studies – from the classical multiple treatment dose studies to up and down / limit dose investigations, in which only animals of one sex, with as few as three animals, have been used in the experiment. The LD50 is the most important data requirement for classification of the product. In the case of agrochemicals, data from the acute oral LD50 is required in one or more rodent species. Classification of hazard is based on acute oral and acute dermal LD50 values from animal investigations. The revised guidelines of LD50 studies...
(OECD, 2002a,b, 2008) have greatly reduced the number of animals used, and researchers employ statistical tools to predict LD₅₀ values. This is the major achievement of the OECD group. In India, too, the Central Insecticides Board (http://www.cibrc.nic.in) banned the conduct of full-fledged LD₅₀ studies as of 2005, recommending instead the use of LD₅₀ studies with the revised OECD guideline using the limited number of animals to conduct the study. Furthermore, the allergy skin sensitization study based on evaluations in Guinea pigs has been replaced with the mouse lymph node assay (OECD, 2010), which employs a minimum number of mice.

4 Extended one generation reprotox testing – Reduction alternative

Reproductive toxicity testing is expected to account for 90% of animals for REACH registration. So far, REACH has received more than 150 proposals with two-generation reproduction data. The International Council on Animal Protection (ICAPo) has called for immediate action by companies and regulatory authorities worldwide to replace the traditional two-generation animal test for reproductive toxicity with a new extended one-generation method. The new extended one-generation reproduction toxicity was accepted by OECD (2011) as the most viable alternative for the two-generation study. The extended one-generation test guideline is designed not only to evaluate reproductive performance but also to assess neurological, immunological, and endocrine integrity in the tested rat. Furthermore, in the OECD 416 two-generation reproductive toxicity study, 2600 rats are used, whereas in the extended one-generation study only 1400 (a reduction of 50%) rats are used and still produce the usual information. Piersma et al. (2011) proved that the one-generation reproduction toxicity study gives good information on the parameters assessed, and the second generation mating and offspring very rarely will provide critical information not seen in the first generation. With an estimated 2000 substances that have to be tested for reproductive toxicity for submission to REACH in the next 3 years, adoption of the extended one-generation reproductive toxicity study would mean a 2.8 million reduction in the number of laboratory animals used (EC, 2006). In August 2011, the OECD officially accepted the extended one-generation reproduction study protocol (OECD, 2011) in place of the existing two-generation reproduction study design. Although the exact in vitro alternatives for many in vivo animal studies may not be possible at this time, some of the replacement alternatives and refinements discussed above must be continued in order to reduce the number of animals in other regulatory studies (http://www.alttox.org).

5 Attention of Regulatory Authorities in India invited

Indian regulators still demand in vivo skin irritation, eye irritation, guinea pig based allergy studies, and two-generation reproductive toxicity study reports on products as dossier. It is time that we abolish these studies and accept the available alternatives, presented above, which are globally accepted. At least in the case of new registrants, repetition of such studies involving whole animals should be discouraged, and risk assessment should be determined with the already available in vivo data. Mere repetition of some of these in vivo studies must be avoided and discouraged, and a more logical approach must be taken. Registration guidelines should be modified to accommodate these practices. Now that India is a member of the OECD GLP system (http://www.indianglp.gov.in), Indian regulators are required to align their test guidelines to OECD guidelines and methods.

Some regulators do not accept test reports that are not GLP (Good Laboratory Practices) compliant, and, therefore, they will ask for repetition of studies that are not GLP. So, if all regulatory in vivo tox studies required are generated under GLP compliance, such test findings will be accepted across the world, thus avoiding repetition of studies and thereby reducing animal use. Since GLP ensures maintenance and experimentation of animals under a humane and ethical environment, the use of only GLP-based studies must be encouraged.

6 CPCSEA and 4Rs

Committee for the Purpose of Control and Supervision of Experimental Animals (CPCSEA) is a body operating under the Ministry of Environment and Forests of Government of India and is responsible for the welfare of animals under experimentation in India. CPCSEA has been actively promoting the concept of 4 Rs (Pereira and Tettamante, 2005) and playing a critical role in the reduction of animals and the promotion of in vitro methods in education, academia, and regulatory testing in our country.

7 Conclusions

The EPA recently launched a program to evaluate 10,000 chemicals for potential toxicity through a program called ToxCast (http://www.epa.gov/ncct/toxcast/). Its goal is to reduce expensive animal studies, turning instead to novel technologies to predict toxicity using stem cells and other non-animal methods.
Thus, research must be encouraged to help scientists search for more such alternatives, with the goal of reducing the number of animals in regulatory studies while still making accurate predictions of toxicity.

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Modern Tools of Species Identification Can Save Millions of Animals Killed in Identification and Animal Taxonomy

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Summary

A large number of animals are taken from the wild every year into practical classrooms for demonstrations of anatomy, physiology, biochemistry, and biosystematics studies. Besides the use of animals in vivisection/dissection, the next large-scale exploitation is in making an animal museum for the study of identification, classification, taxonomy, and phylogeny. A large number of animals are collected regularly from nature for making museum specimens, irrespective of their IUCN status. Over-exploitation of animals in such studies has resulted in almost complete removal of many species from nature. Although many digital alternatives have come into existence in the recent past for the replacement of animals in vivisection, dissections, physiological, and biochemical exercises, very few efforts have been made in search of alternatives biosystematics studies. High-resolution photographs of animals with close-ups of some of their key features, with a biometric database and digitization associated with bioacoustics and molecular characteristics can convincingly replace animal killing in biosystematics studies as well.

Keywords: biosystematics, biometric database, bioacoustics, sonotaxonomy

1 Use of animals in teaching and research

A large number of animals are used in teaching and research all over the world. This has created serious problems for the existence of many species. Laboratory dissection or vivisection has been a common practice in Zoology since the 1920’s as a way of demonstrating the internal organization of an animal. In physiological and biochemical tests as well, the complete animal often is used to study a few parameters. Such practices in Zoology laboratories have resulted in severe loss of biodiversity because greedy animal-catchers often totally sweep large numbers of animals from their natural habitats. Generally, most animals used in the practical exercises are wild-caught species such as *Pila globosa*, *Hirudinaria* sp., *Echinus* sp., *Scoliodon laticaudus*, *Hoplobatrachus tigerinus*, *H. crassus*, etc. Some species are even listed under Schedule I (*Scoliodon laticaudus*) and Schedule IV (*Hoplobatrachus tigerinus*) of the Wildlife (Protection) Act of 1972 in India. Over-exploitation of these and other species in vivisection and dissection, combined with other reasons, has resulted in almost complete removal of several species from nature. The best example of such a loss is *Sara hardwickii* (*Uromastix hardwickii*), which we report from Rajasthan. This lizard used to be a common animal for the dissection tray in many institutions until it was realized in the 1980’s that it had disappeared almost completely from most parts of India.

After animal dissection, the next most important aspect in Zoology in which large numbers of animals are collected by the animal suppliers is to make museum specimens for the study of classification, taxonomy, and phylogeny (biosystematics) (Anderson, 1965; Knudsen, 1966; Longair et al., 1991; Miller and Nagorsen, 1992). This process generally involves exhibiting formalin-fixed or resin-embedded animals (Henry et al., 1997). A large number of animals are collected from nature regularly to make museum specimens, irrespective of their IUCN status. A large number of Zoology museums are maintained in institutions with millions of animals in formalin-filled glass jars. In conventional biosystematics studies, for identification of a species, hundreds of individuals are killed and preserved. Although many digital alternatives have been developed for the replacement of animals in vivisection, dissections, physiological and biochemical exercises, little effort has been made to search for alternatives in biosystematics (Sharma, 2009; Sharma et al., 2011a).

We have found that high resolution photographs of an animal with close-ups of some key features, combined with a biometric database, and their digitization and bioacoustics and molecular characteristics can convincingly replace animal killing in biosystematics studies (Sharma et al., 2011b).
2 Species identification using diagnostic features of morphology with high resolution imaging (HRI)

The morphological and morphometric observations of individuals using images of certain diagnostic morphological features can help in identification up to species. For example, four species of genus Hemidactylus of family Geckonidae, namely H. flaviviridis, H. brooki, H. leachianus, and H. triedrus can be identified with the HRI of their lamellae, skin, and toes. Similarly, frogs of order Anura, Duttaphrynus stomaticus and D. melanostictus can be identified with the help of HRI of their warts of the head region. Among birds the long tailed shrike (Lanius schach), southern grey shrike (Lanius meridionalis) and brown shrike (Lanius cristatus) are good examples. High-resolution imaging can be done conveniently using optical and digital zooming facilities available in almost all still- and video digital cameras.

3 Molecular tools

The recent demonstration of the efficacy of “DNA barcoding” using the cytochrome c oxidase I (COI) gene in correctly identifying specimens of a large number of species of birds and moths provides hope for an unambiguous and standard method of species identification at the molecular level (Hebert et al., 2003). In addition, the studies by Hebert et al. (2003, 2004) also demonstrate for North American birds and for three moth families the concordance in species boundaries implied by molecular phylogenetic versus classical taxonomic methods based on morphological and behavioral characteristics. If this turns out to be the case for a large number of taxa and for groups of closely related species, it opens up the possibility of creating databases that allow identification using independent data sets. For example, parallel identification keys based on molecular, morphological, and/or behavioral data may be developed.

Recent developments in genomics have identified many marker genes. About 26 marker genes have been identified and may be used in biosystematics studies. The technique for identification of these marker genes is based on isolation and characterization of DNA from a few cells – without killing the animals (Sharma, 2010). The cells obtained from the surface of the body or from a drop of blood can be used to produce DNA copies by PCR for partial or complete sequencing using an auto-sequencer. Such information can be used for identification of species.

4 Bioacoustic tools: Sonotaxonomy

Field identification methods involving acoustic sampling for taxa such as birds, frogs, and crickets, and visual sampling based on diagnostic morphological characters are rapid and inexpensive, and they can be developed to be accurate (Riede, 1993). Morphological characteristics often are found insufficient for the identification of cryptic species. Several cryptic species of anurans display a high level of morphological similarities that often make them virtually impossible to distinguish on the basis of morphological parameters. The taxonomic status of some very poorly known groups of frogs of the family Dicroglossidae from the central Aravalli ranges of Western India and the family Microhylidae from the southern parts of India, is assessed by means of acoustic and statistical analyses of differences in temporal parameters of advertisement calls, such as the number of pulses and the call duration, as well as a spectral parameter, dominant frequency, harmonics, peak frequency, amplitude, and power, etc. As these species usually are misidentified or ignored because of their taxonomic complexity in both ecologically diversified regions, we have found bioacoustical diagnosis for each species in order to facilitate identification in the field. Differences in acoustic parameters support the specific status of Sphaerotheca breviceps, S. rolandae, Microhyla ornata, and M. rubra. Populations from these distinct biodiversity regions can be recognized by distinctive advertisement calls, usually corresponding to a recognized species.

The individuals of family Dicroglossidae (S. breviceps and S. rolandae) and Microhylidae (M. ornata and M. rubra), being sympatric species, show great similarities in their morphological characteristics as well as eco-biological needs, but their advertisement call characteristics analyzed using sound analysis softwares, viz., Raven, Avisoft, and Sound Ruler, are very different and species-specific, and they are very useful, particularly in field identification and monitoring (Sharma, 2005). Furthermore, identification and monitoring of species using bioacoustics tools is a humane approach that avoids unnecessary killing of animals.

4.1 Sound analysis system

The call analysis system includes the following steps: Transmitter > Medium (air) > Receiver. The transmitter, e.g., a male frog, emits the sound, which is transmitted through the medium, usually the air, as longitudinal pressure waves. The receiver processes the sound and presents the waves as visual spectrograms that are used for the identification of species. The system of call analysis includes:

1. Recording
2. Storage and conversion into a proper format
3. Generation of a spectrogram
4. Analysis of spectral pattern and development of classifiers

Investigation of animal sounds includes signal recording with electronic recording equipment. Due to a wide range of signal properties and media they propagate, specialized equipment is used instead of the usual microphones. Video cameras are used for the confirmation of the call of a particular species. A sound bank is prepared to store calls in a format applicable to the software. Specific computer programming is designed for the
storage and analysis of recorded data, and specialized sound analyses are used for describing and storing signals according to their intensity, frequency, duration, and other parameters. Before the analysis of an unknown call the software is calibrated with the help of audio-frequency generators. All the sound signals are analyzed on the same frequency and time-scale to ensure that the recorded sound belongs to a different species or the same species.

Data collection involves two main components: sampling followed by processing. Sampling depends on rate and resolution. Rate should be greater than twice the highest frequency to be sampled. Resolution depends on processor intake, i.e., 8-bit or 16-bit. The resolution of 8-bit code is 256 combination steps and that of 16-bit code is 65,536 combination steps.

Most graphical display devices present sound as a time domain feature. The time domain display of sound waves has limitations in analysis. Frequency domain representatives of spectrum are an improved display system achieved by Fourier transform that provides better opportunities for sound analysis. The Fourier transform is a mathematical function that converts the time domain forms of a signal (produced by most measuring and graphical display devices) to a frequency domain representation or spectrum. The input to the DFT is a sequence of digitized amplitude values \((x_0, x_1, x_2, ..., x_{N-1})\) at \(N\) discrete points in time. The output is a sequence of amplitude values \((Ao, AI, A2, ..., AC N/2)-1\) at \(N/2\) discrete frequencies. The highest frequency, \((N/2)-1\), is equal to half the sampling rate \((=1/(2T))\), where \(T\) is the sampling period. The output could be plotted as a magnitude spectrum. Frequency composition of a signal changes over time and can be plotted as a sound spectrogram using spectrum generation and analysis software (Avisoft SAS PRO, Raven 1.4, Sound Ruler). The spectrograms produced by sound plot have frequency on the vertical axis versus time on the horizontal; the amplitude of a given frequency component at a given time is represented by color combinations as per the parameters of sub-menus of the software.

4.2 Micro-scale analysis of calls

Furthermore, microanalysis of a spectrogram could be achieved by slicing the spectrogram (Raven 1.4). A spectrogram slice view is a plot of relative intensity versus frequency at a particular point in time within a signal. A spectrogram slice represents a vertical cross section through a spectrogram at a single time, but rotated 90° so that the frequency axis is horizontal. In fact, a spectrogram is built of a series of spectrogram slices stacked side by side (with their frequency axis running vertically). Whereas a spectrogram view shows a series of slices at successive points in time and represents power at each frequency by a color (by default, grayscale) value, a spectrogram slice view shows only one slice and represents power at each frequency on a line graph. Using a sound ruler, pulse rate, call rate, dominant frequency, fundamental frequency, etc., are recorded. Classifiers and filters are used to sort particular elements or symbols of a call. Generally, sound spectra are discrete in most species, and such diagnostic characteristics of the spectral pattern can be used for the identification of a species. Sound-based identification, classification of call, and spectral parameters can be developed as a strong tool in taxonomy as sono-taxonomy. Sonotaxonomy may be used independently for the identification and categorization of various taxa or as a supporting tool to the conventional taxonomy.

5 Conclusion

High-resolution imaging associated with molecular and sound characteristics can be used not only for the identification of a species but also to resolve confusion among the cryptic species that look morphologically quite similar – without killing the animal.

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Invertebrate Alternatives for Toxicity Testing: Hydra Stakes its Claim

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Summary
The use of vertebrate models in toxicity testing is often challenged for scientific, ethical, and philosophic reasons. Hydra, a freshwater cnidarian, may prove to be useful in this regard. Its simple, transparent, bilayered body allows all its cells to be in contact with the medium, making it a sensitive environmental indicator. The response of hydra to toxicants includes alteration in tentacle morphology, loss of tentacles, change in contractility, altered rate of budding, loss of regenerative capacity, inhibition of gonad formation, inability to attach to substratum, altered behavioral patterns, and mortality indicated by disintegration. Availability of the genome sequence of hydra has expanded the scope of toxicity testing, making this system more versatile. However, the simplicity of this organism, while being its strength, also is its limitation, since its physiology is much less complex than that of vertebrate animals. It is a very good alternative model for preliminary toxicity screening and can substantially reduce the use of vertebrate animals.

Keywords: hydra, xenobiotics, alternative model system

1 Introduction
A wide range of chemicals present in day-to-day life, including petroleum products, petrochemicals, surfactants, pesticides, pharmaceuticals, medicines, household products, food additives, agricultural run-off, by-products of farming, industrial wastes, etc., are released into the environment in large quantities. However, very little information is available about their effects on living and non-living components of the environment. For instance, several studies have shown accumulation of pesticides in groundwater in the original form (Zaki et al., 1982; Gooddy et al., 2001) or in the form of their equally or more toxic end products (Kolpin et al., 2001). Around 80-100 pharmaceutical chemicals and their metabolites have been measured in both effluent and surface waters in several countries (Fent et al., 2006). Exposure of the public to inadequately tested drugs or environmental agents has resulted in several notable disasters. These include severe toxicity from the use of arsenic to treat syphilis, severe birth defects in children resulting from pregnant women using thalidomide, etc. (Melchert and List, 2007).

2 Assessment of toxicity
Toxicity testing is essential for limiting, if not eliminating, chemical pollution of the environment and associated health hazards. Assessment of contamination can be done chemically. However, their short-term and long-term effects on biological systems are of prime concern for maintaining the health of the environment. Toxicity tests on model animals are conducted to evaluate these chemicals for their potential to cause short-term damages such as maiming or death of the exposed individual or long-term damages including heritable or non-heritable mutations, cancer, birth defects, and other adverse effects.

Action of one toxic agent can be altered to different degrees by exposure to other agents. Different environmental pollutants may interact with each other to generate additive, synergistic, or antagonistic effects. An apparently nontoxic chemical, when combined with another substance, either nontoxic or mildly toxic, may turn into a highly damaging agent. The possibility of interaction of test chemicals with other environmental factors, such as diet, temperature, humidity, other stressors, and infectious agents needs to be considered while assessing their deleterious effects (Gardner, 1979). Further, the extent of toxicity of one or a set of chemicals depends on factors such as the concentration of the toxicant, properties of the toxicant, stability and bioavailability of the chemical, exposure time, and such environmental conditions.
as temperature and pH of the medium, as well as susceptibility of the exposed organisms to the toxicant (Babich and Stotzky, 1983).

3 In vitro and in vivo toxicity testing: Advantages and limitations

Measuring the extent of toxicity can be done in vitro or in vivo. In vitro assays are fast, cheap, and ethically more acceptable alternatives to animal tests. Miniature in vitro systems are used to generate quantitative data for the dose that produces a certain biological effect, but their use in predicting in vivo effects is still limited. The exact concentration of the contaminating chemical to which cells are exposed in vitro is difficult to assess. Moreover, the amount of test compound added to an in vitro test system may not be the bioavailable amount in an in vivo test system, and it can vary between test conditions and test compounds depending, for example, on the total cell number, the total protein content in the test medium, and the volatility and hydrophobicity of the compound. Serum content of the cell culture medium is known to vary between laboratories, and this can lead to a different outcome of the same test in different laboratories (Clemedson et al., 2003). Such a variety in outcome complicates a comparison of the damaging potency of a biologically active chemical (Heringa et al., 2004). Due to their low cost and sensitivity, in vitro toxicity tests are beginning to be used more widely as exploratory tools in toxicity studies. In vitro bioassays have been used to investigate the toxic properties of homogeneous particle mixtures, including residual oil, fuel ash, urban air particles (UAP), inert titanium dioxide, elemental carbon, and diesel particles (Carter et al., 1997; Long et al., 2001). Although in vitro cytotoxicity assays have been used for many years, these data have been utilized only to a small extent in drug development because of their limited capacity to predict in vivo toxicity (McKim et al., 2005). In vitro assays have been proven to be valuable for preliminary screening of pollutants with possible biological effects that need to be ascertained in bioassays (Long et al., 2001; Monn and Becker, 1999).

Most reliable data regarding in vivo toxicity is obtained primarily in three ways: by the study and observation of people during normal use of a toxic pollutant or from accidental exposures, by experimental studies using animals, and by studies using cells (human, animal, plant). Among these, human studies are the ideal, but they are very challenging and are limited. Knowledge of the toxicity of xenobiotics to humans is derived by three methods: clinical studies where chemicals are administered to human beings under strictly controlled conditions, epidemiological studies that involve observation of people who are accidentally exposed to chemicals during their life or occupation, and through adverse reaction reports from clinicians in volunteers or patients using the drugs. Generally, toxicity found in animal studies is expected to occur with foreseeable frequency and severity in humans. Differences need to be determined with clinical tests in humans. Animal tests for toxicity are conducted prior to human clinical investigations as part of the non-clinical laboratory tests of pharmaceuticals. Moreover, not all chemicals can be tested in humans; e.g., pesticides and industrial chemicals. In such cases, animal test results often represent the only means by which toxicity in human accidental exposure can be effectively predicted.

4 Selecting a model system

Advantages of animal tests include precisely controlled chemical exposure, well-controlled environmental conditions, and a wide range of toxic effects that also can be evaluated as an ease study of mechanisms of toxicity. Selection of species for toxicity testing depends on the toxicity test to be performed. No single species of animal is sufficient for all toxicity tests. Moreover, it may not be possible to use the most suitable animals, such as primates, for testing because of animal welfare or cost considerations. For example, use of monkeys and dogs is restricted to special cases, even though they represent the species that may react most similarly to humans. Rodents and rabbits are the most commonly used laboratory species due to their availability, low costs in breeding and housing, and comparatively higher predictability of risks to humans from the results obtained. To make the results more reliable, the route of exposure is kept comparable to that of human exposure. The age of test animals should relate to that of humans. Young adults, newborns, or pregnant animals are used depending on the human population that is at potential risk of exposure to the chemical in question. Dose levels normally are selected so as to determine the threshold as well as the dose-response relationship. Usually, a minimum of three dose levels is used. Toxicity can be measured at different endpoints. LC50 (lethal concentration for 50% of the test population) and EC50 (effective concentration at which 50% of individuals are dead) are measures of the severity of toxicity. Chronic toxicity is measured in terms of LCx/ECx, which is the concentration lethal for a small percentage showing an effect of x percent; while LOEC is the lowest observed effect concentration, and NOEC is no observed effect concentration. These measures of toxicity allow one to identify the mode of toxicity and its possible amelioration.

Toxicity effects are measured at various levels. These include:
- Acute Toxicity: Generally the first tests conducted. They provide data on the toxicity from a single brief exposure.
- Subchronic Toxicity: Used to estimate the toxicity from repeated exposures for up to several months.
- Chronic Toxicity: Employed to find the toxicity from exposure for a long duration. Tests can be carried on for years, and they involve larger numbers of animals per test.
- Reproductive Toxicity: Intended to determine the effects of substances on gonadal function, conception, birth, and growth and development. This uses an even larger number of animals and can extend longer than chronic testing.
– Developmental Toxicity: Developmental toxicity testing detects the potential of substances to produce embryotoxicity and birth defects.
– Carcinogenicity: Carried out to test the potential of a chemical to cause cancer.
– Genetic Toxicity: Genetic toxicity is determined using a wide range of test species including whole animals, microorganisms, and mammalian cells. A large variety of tests have been developed to measure mutations and chromosomal damages.

5 Why and how of ethics and possible remedies

All these approaches involve exposure of animals to a test agent and observation for signs of toxicity for a specific period of time. If toxicity is produced before the end of the scheduled study, it may result in animals experiencing pain and distress as a result of localized tissue damage such as eye or skin irritation or systemic toxicity involving damage to various tissues and organs. Pain and distress also may result from the development of neoplastic and chronic disease, as well as from the development of infections in unprotected animals during vaccine testing. For these reasons, the use of animals in toxicity testing has been the subject of ethical concern for many years, most notably in toxicology and biomedical studies. Inflicting suffering on animals solely to benefit humans poses an ethical dilemma. One way to address these concerns before conducting animal testing is to first assess whether the purpose of the experiment justifies the use of animals. If the purpose is found to be justified, the likely pain, distress, and suffering that might be caused to the animals needs to be assessed. These concerns focus on the use of vertebrates in toxicity studies, since these animals are considered sentient beings.

The principles of Replacement, Refinement, and Reduction of the use of animals in research (popularly known as 3Rs of animal testing) (Russell and Burch, 1959) are guiding principles for the use of animals in research to ensure that the suffering of the model animal is reduced to the barest minimum, if not eliminated.

Replacement: The use of non-animal methods such as cell cultures, human volunteers, and computer modeling instead of animals to achieve a scientific aim. If animals cannot be replaced at all, sentient animals should be replaced with non-sentient animals or less sentient ones.

Reduction: The use of methods that enable researchers to obtain comparable amounts of information from fewer animals, or more information from the same number of animals.

Refinement: The use of methods that alleviate or minimize pain, suffering, or distress, and methods that enhance animal welfare for those animals that cannot be replaced.

Different strategies can be followed to implement the principles of the 3Rs. These include:

a) Avoid unnecessary repetition of experiments and use alternatives such as online databases.
b) Use mathematical and computer modeling, e.g., molecular modeling for drug design, predicting biological activity as well as possible side effects of the drug; pharmacokinetic modeling for predicting effects of drugs/xenobiotics and their metabolites by integrating species-specific physiological parameters; partition co-efficient of the chemicals and metabolic parameters.
c) Use in vitro methods, including sub-cellular fractions, tissue-slices, cell suspensions and perfused organs, and cultured tissue from human and other sources in toxicity testing, and for preliminary screening.
d) Use lower organisms with limited sentience and/or not protected by legislation controlling animal experiments. These include such invertebrates as Drosophila, Caenorhabditis elegans, earthworm, hydra, plants, and bacteria (e.g., Salmonella). Some of these can be used as a pre-screen system, especially for agrochemicals and environmental pollutants, genotoxins, and endotoxin detection.
e) Use early developmental stages of vertebrates such as frogs, chickens and mammals for detecting reproductive toxicity, teratogenicity, as well as to study mechanisms of teratogenesis.
f) Use human tissues and volunteers, wherever possible, to avoid the problem of inter-species extrapolation from animals to humans.

6 Hydra as a potential alternative model system

In this context the term alternative refers to any technique that replaces the use of animals, reduces the need for animals in a particular test, or refines an existing technique to reduce the suffering endured by the animal (Rowan and Goldberg, 1985). Replacement of one species with another, particularly a vertebrate with an invertebrate, is considered to be a better alternative. Invertebrates are less likely to raise societal concern compared to higher or even lower vertebrates. When microorganisms, cultured cells and tissues, and in vitro methods are unsuitable replacements for animals, invertebrate models are preferred. For instance, use of a lysate of horseshoe crab amoebocytes is simpler, more rapid, and more sensitive than the corresponding vertebrate test involving the rabbit for pyrogenicity testing.

Invertebrates have been used for decades in acute and chronic toxicity tests for hazard identification. Invertebrates can be very efficient screening systems, even though they cannot entirely replace vertebrates in toxicity testing because barriers and diversity in physiology, biology, and genetics in processing different chemicals and stimuli have not yet been overcome.

Hydra, a simple metazoan animal found in clean slow-moving fresh waters, is very useful in toxicity assessment (Karntanut and Pascoe, 2000). It belongs to phylum Cnidaria, class Hydrozoa and has a simple cylindrical body, a head with hypostome, and a mouth surrounded by tentacles at one end, and a foot and basal disc at the other end (Campbell, 1967) (Fig. 1). The body wall
of hydra consists of two cell layers, ectoderm and endoderm, separated by a collagenous acellular layer called the mesoglea. Epitheliomuscular cells are the main structural components of the body wall. In between the epithelial cells, all the other cell types, including nerve cells and the totipotent stem cells called the interstitial cells or I-cells are located. I-cells occur in the gastric region only. They can give rise to nerve cells, nematocytes, gland cells, mucous cells, and, in the sexual cycle, to oocytes and sperm cells. The nerve cells are organized in a nerve net with condensations to ganglion-like structures in the head and in the foot region (Bosch and David, 1987). Developmental processes are permanently active in hydra, as the cells of the body column continuously divide and get displaced toward the extremities, namely, the head/tentacles and the foot. Here, they differentiate in a position-dependent manner and eventually are shed (Bosch and Fujisawa, 2001; Steele, 2002; Bosch, 2003; Bode, 2003). Hydra is considered to be a good model to assess the toxic effects of water pollutants since it is very sensitive to xenobiotics and metal contaminants present in the medium. In addition, the low costs, efficiency, ease of assay, and availability of large clonal populations permit it to be an effective tool for risk assessment in toxicological studies.

Hydra has an unlimited capacity to regenerate lost body parts (Bosch, 2007). Dissociated cells, when recombined, can self-organize and form a normal, fully intact polyp within two days (Gierer et al., 1972). Under normal conditions individual adult polyps do not increase in size despite continuous cell division in the middle regions and migration to either ends, since growth is perfectly balanced by loss of tissue in the form of buds in the lower gastric region and by shedding of cells at the ends of the tentacles and the basal disk. This combination of uniform growth and local cell loss leads to continuous movement of tissue up the body column into the tentacles or down into the buds and basal disk. Hydra has an asexual mode of reproduction by budding. Its constantly active patterning processes are due to the presence of three continuously dividing tissue-specific types of stem cells, viz., ectodermal and endodermal epitheliomuscular cells and interstitial stem cells. Budding appears to be a survival mode since hydra reacts to environmental stressors such as UV through an enhanced rate of budding (Ghaskadbi et al., 2005). Under certain conditions, hydra can produce gonads (Fig. 1) and undergo sexual reproduction.

7 Toxicity testing using hydra

Since all the cells of hydra are distributed in only two layers, every cell is constantly bathed in the surrounding medium and exposed to the immediate environment. This makes hydra very sensitive and susceptible to minute amounts of environmental toxicants. Studies in the Indian species of the hydra Hydra vulgaris Ind-Pune (Reddy et al., 2011a) show that it takes only a few minutes to alter the cytoskeleton and cell surface features of hydra by exposure to taxol and cytochalasins (Ghaskadbi and Mulherkar, 1984; Chaugule and Ghaskadbi, 2006). Because of

Fig. 1.: (A) Photomicrograph of a live specimen of Hydra vulgaris Ind-Pune with its major body parts labeled. (B) Photomicrograph of a live specimen of Hydra vulgaris AEP strain with an oocyte (arrow). (C) Photomicrograph of a live specimen of Hydra vulgaris AEP strain with multiple testes (arrows). (D) Photomicrograph of a live specimen of Hydra viridissima with a single oocyte (arrow). (E) Photomicrograph of a live specimen of Hydra viridissima with a testis (arrow). The green color of Hydra viridissima is due to the symbiotic unicellular alga Chlorella. Scale bar = 1 mm.
the ability of hydra to asexually produce a clonal population rapidly through budding, it is possible to have a large population available for testing, making the bioassay highly reproducible. Ease of maintenance, simplicity of assay, and high reproducibility have contributed to the popularity of hydra as model organism in acute and chronic toxicity tests of water-soluble compounds (Lum et al., 2003).

Hydra has long been used in classical ecotoxicological testing. The rapid rate of asexual reproduction of hydra by budding allows the effects on population growth of a potential toxicant to be determined in the laboratory. It also provides a rapid, sensitive, and precise approach to the measurement of environmental pollutant effects on freshwater invertebrates (Stebbing and Pomroy, 1978). Hydra is known to be sensitive to both metal and organic contaminants (Stlooff et al., 1983). Deleterious effects on morphological features of hydra were noted due to exposure to the heavy metal pollutant cadmium at ppm concentrations (Kar and Aditya, 2007). This study also provided a random scale to measure the extension of pollution of fresh water in an outdoor setting. In a unique study, the advantages of symbiosis were revealed when endosymbiotic green hydra (Hydra viridissima) and asymbiotic brown hydra (Hydra oligactis) were exposed to aluminium sulphate. Aluminium toxicity triggered mortality, morphological, behavioral, and DNA damage in both the species. DNA damage was greater in brown hydra than in green hydra, however, while behavioral responses to the presence of aluminium ions were observed more rapidly in green hydra. The toxicity also affected reproduction. Brown hydra was more susceptible to aluminium than green hydra, confirming the evolutionary advantage provided by symbiosis (Kovačević et al., 2007). However, it should be noted that H. viridissima was found to be more sensitive to copper and cadmium than H. vulgaris and H. oligactis, while all of them were equally vulnerable to zinc (Karntanat and Pascoe, 2002). In an effort to validate a protocol using new sublethal endpoints of feeding behavior and the ability to attach to substratum, Hydra attenuata polyps were exposed to increasing concentrations of the heavy metal pollutant cadmium chloride. Efficiency of prey capture, ingestion, and attachment impairment were compared to those of morphology and reproduction. These endpoints were much more sensitive to lower concentrations of toxicants than the lethal endpoints. A significant decrease in prey ingestion capacity was seen at lower concentrations of cadmium chloride where capacity to capture prey was unaffected. Ability to attach to the substratum was found to be very sensitive to pollutant concentration, significantly more than reproduction (Quinn et al., 2007). Sensitivity of hydra to extremely minute concentrations (ppm to ppt levels) of organophosphate nerve agents has made it an efficient biosensor. The Hydra bioassay is proposed as a prescreening tool in determining the toxicity of related organophosphorus nerve agents, as well as individual stereoisomers that are yet to be screened for toxicity (Stebbing and Pomroy, 1978).

Dispersants are used for reduction of the toxicity of oil spills to surface animals, birds, and mammals. Along with the oil, however, dispersants themselves were found to be toxic to freshwater organisms (Vindimian et al., 1992). Some of the dispersants were found to decrease the growth rate in green hydra at sublethal concentrations (Mitchell and Holdway, 2000).

In a comprehensive study, acute and chronic toxicity of ten commonly prescribed drugs were assessed using Hydra vulgaris, and some of them were found to adversely affect the regenerative capacity on chronic exposure concentrations (Pascoe et al., 2003). In a similar study, eleven pharmaceuticals and their solvents were classified as toxic, harmful, and non-toxic based on acute and chronic exposure assays using hydra. Acute effects involved morphological changes, while chronic effects were measured using feeding behavior, growth, and attachment as parameters (Quinn et al., 2008).

Hydra polyps are reported to be highly sensitive to short-term exposure to endocrine disruptors such as 4-nonylphenol (4-NP) at concentrations normally found in contaminated sites, but not at those concentrations reflecting lower levels of environmental contamination. Within the first hour of exposure to 4-NP at lethal concentrations, apoptosis was induced, indicating rapid effects of the chemical, and at a lower concentration, loss of tentacles and consequent impaired feeding was reported (Pachura et al., 2005). When exposed to drugs that induce oxidative stress, hydra responds with activation of antioxidant defense mechanisms and metabolic pathways (Quinn et al., 2004).

Effects of reproductive hormone-mimetic compounds found as ecotoxins have been studied in some invertebrates, including hydra. One of these is bisphenol A, a weak estrogenic compound (Krishnan et al., 1993) that is contained in a variety of matrices used for food and drink containers and for dental sealants; it is found in sewage water as well. It interfered with asexual and sexual reproduction of hydra, but only at doses much higher than those detected in freshwater environments (Pascoe et al., 2002; Fukuhori et al., 2005). Data from a hydra regeneration assay was easily and directly correlated with those from vertebrate in vivo teratogenicity and, therefore, the hydra regeneration assay is proposed to be an effective screening tool for the teratogenic potential of various toxicants (Wilby et al., 1990).

Hydra has been found to be a suitable model for testing the biological effects of colloidal semiconductor nanocrystals, the latest biocompatible materials being introduced in biology and medicine. Hydra, treated with quantum rods, showed a subtle tentacle writhing activity that was shown to be calcium-dependent. Results from these assays indicated that the interactions between living organisms and newly synthesized nanomaterials need to be more thoroughly investigated before they are employed in any new nanostructure for such biological purposes as cell-tracking studies, drug delivery, etc. (Tino et al., 2011; Tortiglione, 2011).

8 Promise of hydra as a toxicity testing model

The presence of three lineages of stem cells, perpetual and regular replacement and renewal of all somatic cells in the body, and a continuously active developmental program
make hydra virtually immortal. It can reproduce asexually, producing genetically identical populations endlessly under normal conditions and using the sexual mode under unfavorable environmental conditions. This model offers a unique opportunity to study short-term as well as long-term effects of various classes of toxicants on development, growth, normal life processes, and senescence.

Hydra can survive even when interstitial stem cells are completely eliminated experimentally. This model provides an opportunity to test various cancer stem cells, targeting therapeutic agents for their effects on the vital processes of the organism. Similarly, developmental toxicity of various chemical pollutants can be studied in budding, regeneration, and embryonic development. Budding, as well as head- and foot-regenerating middle pieces of hydra, are distinct and unique systems where all the pattern-forming processes are active without the early embryonic cues. This can provide a good model to investigate the effects of teratogenic pollutants that affect patterning in higher vertebrates. Constantly, recycling somatic cells can be the targets of the molecules that induce apoptosis and necrosis, and their modes of action can be studied in this system. Also, any sign of senescence induction in hydra is certainly because of the chemical being tested since there is no natural aging in hydra.

Until now, toxicity related gene-expression studies have been limited mainly to traditional model organisms whose genomes have been sequenced, such as Drosophila or the mouse. However, the recently published whole genome sequence of hydra (Chapman et al., 2010) has made it possible to use hydra in innovative ways in toxicity testing. This allows the use of gene expression profiles to identify a battery of toxins that pose a risk to the environment or to human health since it is quicker and cheaper than testing on mammalian models. A large number of hydra genes have been discovered since its genome sequence has become available, including unexpected ones such as the neural tube patterning gene noggin (Chatterjee et al., 2001; Chandramore et al. 2010; Chandramore and Ghaskadbi, 2011), the gene for photosensory protein opsin (Plachetzki et al., 2007), the oncogene Myc (Chapman et al., 2010), several genes involved in vertebrate development (Frobius et al., 2003; Reddy et al., 2011b; Hoffmeister-Ullerich 2007), and in innate immunity (Augustin et al., 2010). The effects of environmental insults on these genes can be studied to derive insights into risks to humans posed by these toxins.

The generation of transgenic hydra (Wittlieb et al., 2006) has enabled the study of toxicity from a molecular and cellular perspective. The effects of over-expression/knock out of any homologous or heterologous genes can be studied in custom-made transgenic hydra lines. A transgenic hydra line has been developed expressing a FoxO transcription factor that mediates cellular responses to stress, including oxidative stress and dietary restriction (Bridge et al., 2010), and this can be used as an effective model to study the effects of heat shock. This line of hydra can easily be used to study the consequences of exposure to other inducing factors.

9 Vision for the future of toxicity testing

Systems biology, bioinformatics, and rapid assay technologies are paving the way to a better understanding of how cellular networks or pathways in the human body carry out normal functions. When important pathways are significantly altered by chemical exposures it can lead to significant morbidity or mortality. The latest developments in toxicity-testing can elucidate the cellular response pathways that can result in such a condition. Such a system can evaluate biologically significant alterations without relying on studies of whole animals. For the foreseeable future, some targeted testing in animals will need to continue, as it is not currently possible to sufficiently understand how chemicals are broken down in the body using tests in cells alone. These targeted tests will complement the new rapid assays and ensure the adequate evaluation of chemicals. Dose-response and extrapolation can enable assessment of the possible relevance of results to whole human systems. While we strive to minimize the unnecessary use of animals in experimentation by finding ideal alternative toxicity testing methodologies and protocols, hydra is emerging as a very powerful model system for toxicity testing at the organismal, cellular, and molecular levels.

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Need for Alternatives for Animals in Education and the Alternative Resources

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Summary
Animal use in education, research, and testing has been a subject of controversy. Undoubtedly, the outcomes of animal use have benefited both man and animals. Science has since made tremendous progress, however, and the advancements in modern technologies have opened up a plethora of sophisticated tools and techniques that potentially can replace the old and outdated methods. The discovery/development of alternatives and the newer, realistic approaches and ideas for the replacement, refinement, and reduction of animal use in education have changed the very face of teaching laboratory exercises in Zoology/Life Sciences. The avenues of animal use and the alternatives in education are reviewed from the perspective of India, with emphasis on modernizing the curriculum for programs in these subjects.

Keywords: vivisection, computer simulations, digital alternatives, CPCSEA, UGC

1 Introduction

Animals have long played an important role in education and research. In higher education, in particular, animals are used to teach systematics, anatomy, physiology, pharmacology, and psychology. Many science courses use dissection to help students understand animal anatomy and also to provide them with skills in medical operation/surgical techniques. The practice of animal dissection in laboratories date back to the late 1800’s. The two aspects of animal killing in science education include dissection and vivisection, for which approximately 170 species of animals are used. Dissection is the exploration of dead animals to study their anatomy and physiology, whereas vivisection is animal experimentation, involving cutting, burning, shocking, drugging, starving, irradiating, blinding, or killing of animals.

Every year, millions of animals – cats, frogs, fetal pigs, grasshoppers, minks, earthworms, rats, mice, dogs, pigeons, lizards, etc., – are dissected or vivisected in schools, colleges, and universities. In most of these cases the animals are captured live from the wild. In fact, to meet the heavy demand of the classrooms a single supplier catches 3000 frogs per month, one report says. The consequent loss of biodiversity is unimaginable and poses a serious threat to the fragile environment. The World Conservation Union reported in 2004 that one-third of all amphibian species around the globe, including frogs, were threatened with extinction. For example, thousands of frogs were collected and killed in India for educational purposes, resulting in a decline of the population of aquatic amphibians such as Rana (=Euphlyctis) hexadactyla and Rana (=Hoplobatrachus) tigrina (tigerinus). People for the Ethical Treatment of Animals (PeTA) have estimated that about 6 million frogs are killed for dissection each year. Similarly, the declining population of Sara hardwickii (Uromastix hardwickii), the spiny-tailed lizard, is notably attributable to their large-scale removal from the wild for the purpose of education and research (Dr Krishan K. Sharma, personal communication). Although habitat loss, pollution, and climate change are the primary causes for the decline of the population of these species, demand for dissection specimens increases pressure on this threatened species.

An appropriate and commonly accepted goal of education is to teach individuals to think independently in an analytical and critical way. To achieve this goal, a teacher must become less of an authority, whose role is to simply pass on information, and more of a facilitator, whose role is to promote questioning, exploration, and synthesis. In addition, teachers – and, more importantly, the education boards, the Boards of Studies, and Academic Councils – should frame syllabi and courses in such a manner that ethical education is disseminated and does not contradict the curriculum. It is ironic that Zoology/Life Science teachers on one hand emphasize the importance of biodiversity/wildlife conservation but on the other hand practice dissection in education (Sathyanarayana, 2009). It has been much debated as to whether animal dissection is a process of learning or skill development. According to Rosse (1995), dissection is a destructive (rather than a constructive) process that destroys many of the specimen’s structures and their spatial relationships, precluding reexamination by the students. Generally, dissection is too focused on the acquisition of facts while failing to teach students to conceptualize and synthesize (Rollin, 1990). In the realm of basic sciences, it provides little learning, and whatever skill is acquired has no relevance to the future careers of the learners (Akbarsha, 2007).
2 The changing scenario

As the widespread use of animals in education and testing simmered, complicated by many side effects, a sense of awareness raised an alarm for a group of people: “the visionary ones.” It was the effort and far-sightedness of these “visionaries” that resulted in the 3R principles. The 3R principles – Reduction, Refinement, and Replacement of animal use in research – were proposed by W. M. S. Russell and R. Burch (1959). The 3R concept, also called “alternatives,” delineates the idea of abolishing unethical, unnecessary, and unscientific experiments in bio-medical research, as well a inculcating a sense of care for and humane treatment of animals.

The 3R concept originally was introduced with respect to research activity, but the idea snowballed, as educators, animal welfare groups, scientists, conservationists, and academics realized the potential of alternatives in education and teaching. Now they have seriously pondered the lack of relevance of killing animals for the sake of knowledge. Historically, plenty of animals were easily available for a lesser number of students, but with the ever-increasing number of schools and colleges, the student population has swollen while animal numbers have declined – the situation has reversed itself (Akbarsha, 2007).

Over the past several decades, educators have begun to question the value of this use of animals. Many reports suggest that there is a strong correlation between the way we treat animals and the way we treat fellow humans. Being instructed to cut into an animal without fear of opposition or retaliation from the helpless animal marks animals as “objects” and thus fosters a sense of devaluing animals.

The enormous strides made by computer science, information technology, and allied fields have changed the very face of the education system. Innovative methods of teaching anatomy, physiology, behavior, and psychology are available in various formats for a variety of species. Computer simulations, three-dimensional (3-D) models, videotapes, cadavers, and other alternatives involve little or no use of animals. This has resulted in millions of animals being saved. Teachers and professors have started recognizing that students can learn equally well through the use of modern technology, and reports published in scientific journals testify to it (van der Valk, 1999).

Teachers play an important role in framing the courses and syllabi, and the fate of hundreds of animals hang on them. There was a time when teachers relied on and preferred animal dissection for the purpose of knowledge dissemination, rebuffing the latest technologies. This could be attributed to an unwillingness to adopt the modern technology or to their lack of awareness/understanding about it. However, effective campaigns, relentless efforts by the welfare organizations, ethical issues, budgetary concerns, and innovative technologies have made an impact. The attitude of teachers has changed or is changing, and they have come forward and proposed substituting the use of computer simulations and multimedia presentations in place of the conventional dissections. Today, the Zoology/Life Science teachers are experiencing an ethical crisis over animal dissections and experiments (Chitralekha, 2009).

3 Laws regarding animal welfare

India has one of the most comprehensive animal protection laws in the world. Detailed codes of conduct govern the use and treatment of animals, both domestic and “wild.” The Constitution of India, section 51 A(g) demands from the citizens as their fundamental duty to protect and improve the natural environment including forests, lakes, rivers, and wildlife and to have compassion for all living creatures.

The Prevention of Cruelty to Animals Act, 1960, Section 17(2) (d) & (f) advocates the rational use of animals in experiments and education. The law clearly states that “experiments on animals are avoided wherever it is possible to do so; as for example in medical schools, hospitals, colleges and the like, if other teaching devices such as books, models, films and the like may equally suffice.”

The Wildlife (Protection) Act, 1972, provides legal protection to all species included under various schedules, i.e., schedule I to schedule VI. Some of the commonly used animals in dissection, such as sharks and rays (Elasmobranchii), Bonnet macaque (Macaca radiata), Rhesus macaque (Macaca mulata), and freshwater frogs (Rana spp.) are protected under these schedules. The ongoing use of animals for dissection/vivisection in education in India is in violation of the legal provisions (Vasudevan and Surpriya, 2011). Following reports of dissection of frogs at a Medical College without permission of the Chief Wildlife Warden, a team of officials from the District Forest Office raided the college premises. The District Forest Officer (DFO) said frogs were found on the premises, and the college authorities did not have permission to dissect them (Tribune India, September 30, 2009). Ignorance of the law is no excuse for those who kill animals.

In addition to these animal welfare rules, The Breeding of and Experiments on Animal (Control and Supervision) Rules, 1998, govern the care and concern about experimental animals, and it is monitored by an independent Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), a statutory body under the Prevention of Cruelty to Animals Act, 1960, in the Ministry of Environment and Forests.

4 Initiatives in alternatives to dissection

The use of alternatives got its due attention and significance when Jenifer Graham, a high school student in California, sued her school for insisting that dissection was the only method it recognized for learning frog anatomy. Following this event, more students objected openly to dissecting animals, which resulted in the genesis of “Choice-in-Dissection-Laws.” These laws made provisions for the students to choose between animal dissection and alternative learning modalities. Today, many countries, including Argentina, Switzerland, Norway, the Netherlands, and Denmark, have enacted legislation to prohibit dissection below the university level, and several other countries do not require it.
Being a country with a human population of more than a billion, India, too, had witnessed many such incidences, and some of the State Education Boards and Universities have acted to shed, partially, syllabi involving animal use in Life Sciences programs (Salunkhe, 2009). The Zoology students of the University of Kerala complained to People for Animal (PfA) trustees regarding the painful killing of frogs by pithing without anesthesia. Pithing involves the insertion of a sharp object into an animal’s skull and moving it around vigorously to “scramble” the brain. This is a common practice for rendering frogs “brain-dead” for physiology experiments. This indeed has traumatizing effects on the students. The PfA forwarded their petition to the Vice-Chancellor, the Registrar, and the Chairperson of the Board of Studies. As a result, all frog experiments were removed from the syllabus from 2005–2006 onwards.

At the same time, the University Grants Commission (UGC) requested all the Universities and Colleges to ensure the strict adherence to the provisions of the Wildlife (Protection) Act 1972 while procuring animals for use in laboratories. At the request of PfA and Pf/A, the University Grants Commission (UGC) constituted an Expert Committee in 2010 to look into the possibility of reducing/dispensing with the dissection of animals in colleges and universities. The Ministry of Environment and Forests (Government of India), New Delhi also asked the UGC to explore the possibility of ending the practice of dissecting animals in laboratories. These initiatives taken by the UGC boosted other organizations, such as the Pharmacy Council of India, which sent letters to all Pharmacy institutions to use computer simulations as an alternative to use of animals. Rather slowly but steadily, the revamping of traditional animal-based programs took place. These laws, regulations, guidelines, and notifications require the educators to use non-invasive alternative methods to replace the killing of animals in the laboratories.

The Medical Council of India, the apex board that decides the course structure and sets laws for medical colleges, has conceded that exemplary and innovative alternative tools are available that can replace or at least lessen the use of animals for the purpose of education. The Council directed the medical schools to replace the use of live animals in medical course experiments with sophisticated non-animal training methods, such as computer-aided learning (Medical Council of India, 2009).

A number of Zoology/Life Science/Medical/Pharmacy and Veterinary Educators in colleges and universities have played a role in preventing the animal killings, improving the learner’s (students’) learning experiences by replacing dissection with modern, effective, and economical non-animal, student-friendly alternatives in the science laboratories.

The biggest tickets to ecstasy for animal welfare, teachers, and scientists were earned when the UGC accepted the Expert Committee’s recommendations and enacted new guidelines, which is historic (http://www.ugc.ac.in/notices/guidelines_animaldissection.pdf). For the first time, animal dissection is almost completely removed from the university courses. This triumph was made possible by the painstaking efforts of many authorities, teachers, scientists, animal welfare organizations and, in particular, MGDC, Pf/A, I-CARE, and PeTA India, to name a few.

5 Alternatives: what are they and where?

Alternatives, in the present context, are defined as educational aids or teaching approaches that replace harmful animal use or complement humane education. Humane education in the Life Sciences is progressive education for which the teaching objectives are met using humane, alternative methods where animals are free from harm and students have freedom of conscience in an education that encourages holistic perception and a respect for life (Jukes and Chiuria, 2003). In their book, “The Principles of Humane Experimental Technique,” Russell and Burch (1959) defined the Replacement alternatives as methods that permit a given purpose to be achieved without conducting experiments or other scientific procedures on animals. The 3R concept has evolved today as “the science of alternatives,” making tremendous changes in laboratory techniques across the world in knowledge; only humane science can be good science. The use of non-animal methods in experimentation is integral to credible research.

Many different types of non-invasive animal alternative resources are available now, including models, manikins, multimedia computer simulators (CD-ROMS), online simulators, cell culture techniques, in vitro toxicology, molecular tools, functional genomics, tissue engineering, systems biology, cell-laden hydrogels, molecular and acoustic (sonotaxonomy) tools, microlabs, and ethically sourced animal cadavers. Field study of animals also is considered one of the best alternatives to dissection.

5.1 Models and manikins

The models are used to study the anatomy and to learn animal handling without animal stress. The three-dimensional plastinated animal models (Plastination is a chemical process that transforms the tissues of a dead animal into plastic) are available. A manikin is an anatomical model. For example, a manikin of a full-size dog has been designed for Cardiopulmonary Resuscitation (CPR) training. This alternative tool is helpful in teaching laboratory exercises in pharmacy and medicine. Manikins have been employed in veterinary education as well. Models of anatomic parts, whole-body manikins, and various computer-based learning programs have provided educators with training tools for students aiming to become professional veterinarians.

5.2 Digitalized CD ROM

The development of e-learning technology, a computer-based technology, has contributed significantly to our knowledge of effective Animal Science education. Students can be trained in animal anatomy using computers, and virtual reality technologies are revolutionizing the educational system. Today
we are in an Information and Communication Technology (ICT) era, and it requires that teachers be both digitally literate and liberally sensitive. The time has come to change the laboratory curriculum and put the available digitalized CD ROM, other animal alternatives, and web resources to good use. Teachers often cite the cost of alternatives resources and computers as a reason for not implementing them, but the alternatives provide great advantages.

The advantages of computer simulations include:
- Students can learn different variables at one time and various parameters on a large or small scale.
- Computers can offer scope for feedback, provide hints, and offer help.
- Experiments can be repeated at any time and almost anywhere.
- Teachers and students can make use of one CD-ROM repeatedly.
- These methods are cost-effective and affordable when compared to the cost of animals (dissection requires multiple animals to be purchased).
- They provide for conservation of animals and balanced ecosystems.
- They enhance the creativity of teachers, as opposed to the conventional dissection process.

5.3 Cadavers
Cadavers are dead animals from a humane and ethical source (for example, animals that died naturally or of illness, injury, accidents, or severe behavioral problems). An animal killed intentionally cannot be considered ethically sourced as it has been subjected to pain and suffering. The importance of ethically sourced cadavers and their implications for teaching and education is vividly described by Martinsen and Jukes (2007). Cadavers are used as alternative educational tools in veterinary colleges to facilitate learning of animal anatomy and to fine-tune surgical skills, especially for novice veterinary students (Tefera, 2011).

6 Conclusion
Dissection is an old-fashioned teaching and learning method that has worn out over the years and is of negligible use in the modern education system. With the advent of modern technology educators should frame curricula that expose students to the acquisition of knowledge through observation rather than through the archaic method of dissecting animals (Sathyanarayana, 2009). Laboratory curricula should be designed to develop the student’s sense of responsibility towards animal welfare, as well as an appreciation of and respect for life. It is high time for universities to seek avenues that will minimize animal use for teaching purposes (Surendran and Easwaramohan, 2009), adhere to the UGC’s new Guidelines regarding animal dissection, and offer alternatives for dissection. It has been rightly pointed out that a commitment by the educators at the national and institutional levels is required to bring changes in the undergraduate curriculum that will result in a reduction of animal use in the laboratory curriculum. Field trips, biodiversity, biosystematics, and behavioral studies should be added to the curriculum to make teaching more interesting and learning more rewarding. Attention of readers is invited to the treatise “The Use of Animals in Higher Education: Problems, Alternatives and Recommendations,” by Jonathan Balcombe, which has reviewed the state of affairs pertaining to alternatives in education (Balcombe, 2000). The weak point, as opined by an award-winning teacher, is “most of us teach the way we were taught rather than the way we learn.” At the same time, alternative tools should be defined clearly and objectives should be set rationally, with a realistic approach to be undertaken regarding the welfare of the animals, the benefit to the students, and compassion for animals overall.

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Scientists in India Sensitized About Systems Biology Approach to Toxicity Testing: Hands-on Workshop on Computational Systems Biology and Dose-Response Modeling

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Computational systems biology is an important tool for prediction and better understanding of toxicity through dose-response modeling. The in silico approach to toxicological studies has the potential to significantly reduce the use of animals for risk assessment, and there is an urgent need to introduce these techniques to the scientific community in India. The first-ever workshop in India on “Computational Systems Biology and Dose Response Modeling” (CSBDRM), in academic partnership with The Hamner Institutes for Health Sciences, Research Triangle Park, NC, USA, was conducted by the Mahatma Gandhi-Doerenkamp Center for Alternatives to Use of Animals in Life Science Education, at Bharathidasan University, Tiruchirappalli, on March 2-4, 2012. The Hamner team included three instructors: Dr Sudin Bhattacharya was present in person, while Dr Melvin E. Andersen and Dr Qiang Zhang gave lectures and hands-on training remotely through the online videoconferencing tool Webex. The workshop participants were drawn from different career stages and profiles: students, teachers, and scientists, and they came from different parts of the country. After an introduction to the theme, scope, and objectives of the workshop by Dr Andersen, the instructors gave lectures on several topics: “Ultrasensitive response motifs in quantitative cell signaling,” “Network biology,” “Cellular homeostasis, stress response, and adaptation: network motif basis for nonlinear, threshold, and hormetic responses,” “Binary decision making in biological systems,” “Network motifs – recurring components controlling biological functions,” “Eukaryotic cell cycle checkpoint control,” and “Stochasticity in gene expression and its implication for dose response.” The application of these varied concepts was demonstrated with two case studies, “Suppression of B cell differentiation by dioxin” and “p53 dynamics and DNA damage response.” The hands-on exercises included: i) an introduction to the Berkeley Madonna® software with a simple model of protein synthesis/degradation; ii) models for reversible binding, the Hill function, zero-order ultrasensitivity, and the MAP kinase cascade; iii) a bistable gene auto-regulation model; iv) a model of proportional negative feedback control and non-linear response; and v) modeling a simple cell cycle circuit and checkpoint control. Dr Andersen wrapped up the proceedings with the talk “Computational biology and dose response modeling and risk assessment: where to from here?” In spite of the limitation that some of the presentations and training had to be given online, the two-way interaction was lively and rewarding. The feedback responses collected from the participants were analyzed and reflected thorough overall satisfaction with the entire workshop. All of the lecture materials and exercises are freely available on The Hamner Institutes’ website. Readers are encouraged to access this material for a more thorough appreciation of the workshop.

Keywords: in silico toxicology, systems biology, dose response modeling, alternatives to animals

1 http://www.thehamner.org/about-the-hamner/education-training/dose-response-modeling/
1 Introduction – In silico toxicology as alternative to animal testing

In view of the growing popularity of the 3Rs concept and the limited value of the use of animals in experiments aiming to look at human endpoints, non-animal methods in toxicity testing and risk assessment are gaining momentum, most specifically in vitro and in silico toxicology. In recent years tremendous progress has been made in computational biology and mathematical modeling. In silico toxicology or predictive toxicology has emerged as a fast and cost-effective alternative technique for early assessment of a plethora of toxic chemicals (Helma, 2005).

In silico toxicity prediction techniques include kinetic modeling (of biochemical pathways relevant to toxicology), expert systems (where information about chemicals and biological systems are given as inputs and algorithms developed to predict the endpoints) and data-driven systems (including SAR and QSAR, which adopt data mining techniques such as K-nearest neighbors, neural networks, Bayesian statistics, and support vector machines) (Helma, 2005). PBBK models incorporating QSAR- and in vitro-derived parameters, combined with in vitro assays of tissue/organ toxicity, can substantially reduce the use of animals in toxicity testing and risk assessment (Blaauboer, 2001, 2002, 2003). Commercial expert systems like TOPKAT and MCASE and the freely available CAESAR, OECD QSAR Application Toolbox and ToxMatch are helpful in predicting toxicity endpoints (Cronin et al., 2001).

2 Systems Biology: A key tool

The major bottleneck in drug discovery is the unforeseen effects of drug toxicity arising from the network properties of the components of a cell. For many years, toxicologists have employed the conventional, reductionist approach by analyzing individual components of biological systems in response to a toxic exposure. Such studies reveal only a portion of the dynamic processes of the system rather than what takes place in a holistic biological system (van Vliet, 2011).

Systems biology has been defined as the iterative and integrative study of biological systems as they respond to perturbations (Auffray et al., 2003). However, it also should be stated clearly that identifying all the different genes, proteins, and metabolites in a biological system does not by itself lead to a systems biology understanding (Ademem, 2005). The challenge is to discover the dynamic interactions between the components on a multi-scale level and how they maintain and control homeostasis. To obtain a system-level understanding of a biological system a number of different properties need to be investigated (Kitano, 2002).

The first step is to identify the structural components of the system, including its networks of genes, proteins, and biochemical pathways. To understand the dynamics in a biological system it is important not only to have quantitative information but also to comprehend the temporal and spatial dynamics of molecular and biochemical processes. This effort can be aided by performing a limited number of small-scale, proof-of-principle systems biology studies to confirm the network structure and dynamic behavior of “toxicity pathways” – normal cellular signaling and developmental pathways that are perturbed by drugs and chemicals (van Vliet, 2011). Typically, cellular developmental networks comprise multiple nodes and feedback and feedforward loops that can generate specialized behaviors such as toggle switches and oscillators, thereby creating a dynamic cascade of sequential activation of sub-components of the network. Integrated systems biology data – gene expression, phosphoprotein alterations, transcription factor analysis – will be needed to provide the dynamics and functional characterization of these networks and their associated toxicity pathways (Andersen et al., 2011). The combination of dynamic principles, mathematical frameworks, and computational modeling techniques makes systems biology an efficient tool for contemporary toxicology studies.

3 Dose-Response Modeling: prediction of cellular dynamics

Computational models in toxicology require integration of the quantitative correlations of exposure (i.e., dose, time intervals, and outcome) (Henry, 2003). Dose-response modeling of toxicity pathways should, ideally, be an iterative process where computer simulations and experimental data inform each other. Incorporating experimental observations back into the model can improve its design and refine its predictive capabilities. Numerical simulation of biological models allows us to mimic the dynamic behavior of molecular circuits and toxicity pathways. The most common approach to modeling dynamic systems is to use deterministic simulations based on ordinary differential equations (ODE) (Zhang et al., 2010). Dose-response modeling is a valuable computational tool that has been used to provide insights into the intricate design and function of molecular circuits underlying a variety of biological processes. Examples include cell cycle regulation, signal transduction, cell differentiation, stress response, and biological rhythms (Bhalla et al., 2002; Forger and Peskin, 2003; Novak and Tyson, 2003; Chang et al., 2006; El-Samad and Khammash, 2006). There are a variety of software tools in which detailed network models of gene/protein signaling cascades can be assembled to simulate time-course and dose-response behaviors (e.g., JDesigner, Jarnac, COPASI, PathwayLab). These programs often include the capacity to vary model parameters automatically to explore their effect on system dynamics.

Two important recent strategies/reports have spurred the interest of toxicologists and other stakeholders: “Mapping of the human toxome” (Hartung and McBride, 2011) and the vision proposed by the U.S. National Research Council (NRC), “Toxicology Testing in the 21st Century” (Andersen et al., 2011). These focus on identifying and mapping “pathways of toxicity” in humans, and subsequently moving away from traditional high-dose animal studies to pathway-based perturbation of cellular
responses using well-designed in vitro assays for low-dose responses. The identification of common pathways of toxicity, and the quantification of the signals therein, should continue to be the major focus of toxicological research. This not only will advance our understanding of complex intracellular networks but also will assist in the development and validation of in vitro and in silico systems. In view of its agility, computational dose-response modeling has the potential to reduce or replace animals in repeated-dose toxicity testing (Stokes and Wind, 2010).

4 CSBDRM – The Indian Scenario

India is a country with a long and rich tradition in science and mathematics, including the practice of indigenous traditional medicines. Both historically and in the modern era, it has contributed important scientific discoveries and concepts to the world. However, scientific practice in India over the last several decades has relied too heavily on animal use in education, research, and toxicity testing. Animal dissections, physiology experiments, and animal toxicity studies are in wide use in the curriculum of medical, pharmacological, veterinary, and life science programs. Also, the country is rich in pharmaceutical, biotechnological, and clinical research industries, all of which rely largely on information gathered from animal experiments.

In recent years, however, attitudes towards animal use have begun to change, and Indian governmental agencies such as the University Grants Commission (UGC), the Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA), and the Indian Council of Medical Research (ICMR) have released guidelines to limit and/or govern animal use in education, research, and testing. This has launched a historic shift that already has spared the lives of millions of animals, and this effort is greatly appreciated by experts, scientists, animal welfare organizations, and other stakeholders. This trend should be adopted in other countries as well. Despite the fact that the Indian scientific community has contributed extensively to non-linear dynamics in physics, mathematical modeling, and concepts such as kinetics and dynamics in chemistry, it has not done as well with the adoption of computational systems biology and dose-response modeling tools in the biomedical sciences.

5 Incitation, prolation, and accomplishment

The Mahatma Gandhi-Doerenkamp Center (MGDC), an academic-based service organization with excellent infrastructure, was established at Bharathidasan University in Tiruchirappalli, India by the Doerenkamp-Zbinden Foundation (Switzerland) in 2009. The Center has introduced systems biology and in silico toxicology into its program as tools for alternatives to the use of animals in parallel to in vitro toxicology. The Center’s mission focuses on replacing, reducing, and refining the use of animals in life science and biomedical science education, drug discovery, and toxicity testing (Akbarsha and Pereira, 2010; Akbarsha et al., 2010). MGDC has established its own network with renowned organizations that can contribute to achieving this mission. The Hamner Institutes for Health Sciences is one such organization. The Center for Dose Response Modeling (CDRM) at The Hamner Institutes performs cutting-edge research to improve chemical and drug safety. As one of the centers of the Institute for Chemical Safety Sciences at The Hamner Institutes, CDRM has the mission of developing computational systems biology tools to facilitate an understanding of dose-response relationships at the cellular and tissue levels for environmental chemicals and drugs. Specializing in simulation of chemical perturbations of intracellular signal transduction pathways and gene regulatory networks, the Center has a particular research focus on inferring the shape of dose-response curves in the low dose region relevant to environmental exposures. Dose-response modeling based on computational systems biology is an emerging field in toxicology and chemical safety assessment, and CDRM is also active on the educational front to disseminate novel quantitative tools among practicing and future toxicologists. The three-day workshop on “Computational Systems Biology and Dose Response Modeling” taught by CDRM scientists provided a rewarding outcome of the collaboration between MGDC and The Hamner Institutes for Health Sciences. To the best of our knowledge, this was the first-ever workshop to be conducted on this topic in India. The workshop was fiscally supported by the Doerenkamp-Zbinden Foundation (DZF), Switzerland, and the Department of Biotechnology, Government of India.

6 Course content

The goals for this short course were for the participants to learn about:

- Current computational modeling techniques for the quantitative investigation of how biological systems respond to perturbations at the cellular level
- Common motifs in signal transduction and gene regulatory networks that underlie systems-level cellular behaviors including homeostasis, adaptation, threshold response, binary, and irreversible cell fate decisions
- How molecular circuits comprising genes and proteins give rise to various nonlinear dose-response behaviors
- The molecular circuits driving the cell cycle and checkpoint control, and its implications for modeling dose-response with respect to cell proliferation
- The noisy (stochastic) nature of gene expression, its biological functions, and implications for the shapes of dose-response curves

2 http://www.mgdcaua.org/
3 http://www.thehamner.org/institutes-centers/center-for-dose-response-modeling/
– Using these techniques and concepts to develop computational tools for understanding and predicting nonlinear dose response behaviors – a vital need for toxicity testing in the 21st century

7 Lectures/Talks

7.1 Computational dose-response modeling: some background
Dr Melvin E. Andersen, Associate Director of The Institute for Chemical Safety Sciences at The Hamner Institutes, launched the workshop with an introduction to CSBDRM by videoconference through Webex. Dr Andersen explained exposure-dose-response relationships using a physiologically-based pharmacokinetic (PBPK) model for styrene, emphasizing the role of computational systems biology in understanding the pharmacodynamic aspect of the exposure-dose-response continuum. Addressing the case of dioxin, he explained how it increases expression of proteins that bind dioxin (CYP 1A2) in the liver, which would sequester the compound in the liver. Such protein induction occurs in specific regions of the liver acinus. He described his previous work developing multi-compartment liver acinus models to explain regional protein induction in the liver. Biologically based dose-response (BBDR) models for cancer and tumor progression were explained. The concepts of positive feedback and binary and graded response in gene networks were introduced.

Taking the example of the oocyte maturation switch, Dr Andersen illustrated how biological components can function together to give rise to switch-like responses. Mitogen-activated protein kinases (MAP kinases) were introduced as a versatile signaling motif. Such motifs often are embedded in intracellular response networks. Quoting from an article by Lander and Weinberg published in Science in 2000 (Lander and Weinberg, 2000), which stated that “the long-term goal” of systems biology is to use genomic information to “reconstruct the complex molecular circuitry that operates within the cell,” and that “a longer term goal … is to create mathematical models of these biological circuits and thereby predict these various types of cell biological behavior.” Dr Andersen emphasized that these goals now appear to be within reach. A systems approach could be developed for dose-response, looking at dose, circuitry, cells, and tissues. Touching upon the 2007 NRC report, Toxics Testing in the 21st Century (NRC, 2007), he derived a possible safety assessment schematic starting from in vitro toxicity results and went on to explain how computational systems biology would be useful in mapping and organizing toxicity pathways by using bioinformatics tools and simulating toxicity pathways as dynamic systems.

7.2 Ultrasensitive response motifs in quantitative cell signaling
Dr Qiang Zhang, Director of the CDRM at The Hamner Institutes, introduced the concept of ultrasensitive response motifs in quantitative cell signaling. The pharmacokinetics and pharmacodynamics of chemical exposure lead to toxicological effects through biochemical changes at the level of individual cells. Cellular responses are mediated by biochemical circuits/networks. There are structural and functional parallels between these intracellular molecular circuits and electronic circuits, and as with the latter, “reverse engineering” can be used for a better understanding of the working of molecular circuits. The components of these intracellular circuits are connected in specific ways to form functional building blocks. “Network motifs” are relatively simple building blocks that frequently appear in complex molecular circuits and possess specific signaling properties. Specifically, ultrasensitive response motifs amplify small changes in input to a relatively large percentage change in output – producing a nonlinear (sigmoid) dose-response curve often modeled by the Hill function. Some common examples of ultrasensitive response motifs were introduced: positive cooperative binding, homomultimerization, multistep signaling, molecular titration, covalent modification cycle, and positive feedback. The MAP kinase cascade was discussed as an example of how combinations of ultrasensitive motifs can generate switch-like responses. Ultrasensitive motifs also are required to generate more complex cellular behaviors such as bistability, robust homeostasis, and oscillation. In the hands-on exercises in the afternoon session (described in Section 8), computational models of a number of these motifs, as well as the MAP kinase cascade, were demonstrated using Berkeley Madonna®.

7.3 Network Biology
Dr Sudin Bhattacharya, Research Investigator at The Hamner Institutes, kicked off his session with an in-person talk on “Network Biology.” He introduced the idea of “canonical pathways,” using the examples of peroxisome proliferator-activated receptor alpha (PPARα) and nuclear factor kappa B (NF-κB) signaling. In most cases, however, the structure of these pathways is not fully understood, and signaling networks have to be reconstructed from genome-wide data. An application of network reconstruction was illustrated with the yeast hyperosmotic stress response (Wang and Chen, 2010). The context-dependent, dynamic behavior of signaling networks has been investigated for several human diseases, for example breast cancer (Chuang et al., 2007) and systemic inflammation (Calvano et al., 2005). Cytoscape was introduced as a network visualization and analysis tool. The inference of transcriptional regulatory networks from gene expression and genome-wide transcription factor location data was discussed with the example of ongoing work on the PPARα pathway at The Hamner Institutes. The concepts of network structure and connectivity, network hierarchy, information flow, network dynamics, and the use of high throughput data sets in network inference, were emphasized.

7.4 Cellular homeostasis, stress response and adaptation: network motif basis for nonlinear, threshold, and hormetic responses
Dr Zhang started the second day of the workshop with a talk on cellular homeostasis, stress response and adaptation, and the
role of network motifs in generating nonlinear threshold and hormetic responses. Homeostasis can be defined as the ability of a biological system to maintain a stable internal milieu in the presence of external fluctuations, and it operates at the cellular, tissue, and whole-body levels. The focus of this lecture was on cellular homeostasis, which is enabled by the adaptive cellular stress response. Examples of cellular stressors, such as starvation, oxidative stress, ischemia, and dehydration, were described. The nature of the adaptive cellular stress response was explained with the example of oxidative stress. Negative feedback is the primary network structure that underlies adaptive stress response. However, feedforward control also can play an important role in this regulatory mechanism. A number of different negative feedback-mediated stress responses were described to show that a general control scheme involving a stressor signal, transcription factors, anti-stress genes, and the controlled variable (e.g., reactive oxygen species or misfolded proteins), underlies cellular homeostasis. Blood glucose homeostasis and sex hormone homeostasis were discussed as examples of negative feedback-mediated homeostatic control at the whole-organism level.

Next, the shape of the cellular dose-response curve in the context of various negative feedback control mechanisms was illustrated. Proportional negative feedback control can produce only partial adaptation, while integral feedback enables perfect adaptation and threshold responses. Several concepts necessary for a quantitative understanding of homeostatic control then were introduced, including the response coefficient, the Hill coefficient, open-loop (local) and closed-loop (system level) gains. Various ultrasensitive motifs such as homo-multimerization, positive feedback, and zero-order phosphorylation can be embedded in a negative feedback loop to generate higher local gains and increase the overall closed-loop gain. In the case of proportional feedback, the combined effects of the loop gain, maximal gene induction, and enzyme saturation can generate a complex dose-response curve that transitions through multiple phases with increasing levels of the stressor and is intrinsically nonlinear at low stressor doses. The generation of perfect adaptation and threshold responses with integral negative feedback control then was explained, accompanied by the examples of high-osmolarity stress response in yeast and adaptation of tumbling frequency to chemo-attractant concentrations in the bacterium *E. coli*.

While negative feedback control can detect and respond to changes in the cellular state, in feedforward control the presence of the stressor itself is directly sensed and used to activate anti-stress mechanisms such as gene induction. Several illustrative examples of feedforward control were discussed—the bacterial heat shock response in *E. coli*, phase I-II cross induction of xenobiotic metabolizing enzymes, and temperature control at the whole-body level. Feedforward control can generate dose-response curves with various shapes: nonlinear monotonic, threshold, and hormetic, depending on the strength of the feedforward mechanism. Thus, cellular homeostatic control is underpinned by regulatory networks organized in negative feedback and feedforward loops. In the following hands-on exercise with Berkeley Madonna®, Dr Zhang demonstrated an integrated model of a negative feedback circuit generating homeostatic response.

### 7.5 Binary decision making in biological systems

Dr Zhang’s presentation was followed by a talk by Dr Bhattacharya on binary decision making in biological systems. He introduced the idea of different “cellular fates,” e.g., proliferation, differentiation, and apoptosis, and used the example of hematopoiesis to illustrate the progressive acquisition of distinct fates (e.g., erythrocytes, eosinophils, and monocytes) in the blood cell lineage. These different cell fates are associated with distinct, stable gene expression profiles that arise from the cellular genome. Specifically, gene-protein and protein-protein interactions generate stable “attractor states” for the dynamic system formed by the network of intracellular molecules. These distinct “attractor states” correspond to different cell fates. The concept of “bistability/multistability,” the existence of two (or more) distinct stable steady states for a system, was explained with the examples of competent/vegetative state transition in the bacterium *B. subtilis* and all-or-none activation of lac operon genes in *E. coli*. A positive feedback loop with an embedded ultrasensitive motif can generate a bistable system. Using gene autoregulation as a canonical model, Dr Bhattacharya illustrated how bistability can produce both reversible and irreversible switch-like transitions between different cell fates, as well as a “cellular memory” mechanism through hysteresis. A bifurcation diagram was derived schematically for the gene autoregulation model as a visual illustration of bistability. Phase plane analysis with nullclines was introduced as an alternative way to understand transitions between distinct cell fates. It was emphasized that bistability does not occur just in bacteria but also in eukaryotic cells. Bistability arising from positive or double-negative feedback loops was illustrated with the examples of adipocyte differentiation, erythrocyte versus myeloid lineage determination, and the generation of distinct phases in cell cycle progression. The hands-on exercise that followed included creation of a simple gene autoregulation model, exploration of its bistable behavior with continuous and pulse input, and phase plane analysis with nullclines to quantitatively derive a bifurcation diagram.

### 7.6 Network motifs – recurring components controlling biological function

The next talk by Dr Bhattacharya elaborated on recurring network motifs and the manner in which they control various biological functions (Alon, 2007). The positive/double-negative feedback loop motif can produce an irreversible or reversible bistable switch, depending on whether the strength of the feedback is high or low, respectively. The negative feedback loop motif, in addition to enabling cellular adaptation and homeostasis as previously discussed, can: (a) reduce the response time in gene expression through autoregulation; (b) reduce noise in gene expression; and (c) generate oscillations. Feedforward loop motifs can be either of the coherent or
incoherent type. Coherent feedforward loops can: (a) detect signal persistence and introduce time delay in a response; and (b) filter out small transient noise. Incoherent feedforward loops, on the other hand, can: (a) generate pulses; (b) accelerate the response time; (c) enable adaptive, homeostatic or hormetic cellular responses; and (d) detect fold changes in an input signal.

Transcriptional regulatory networks typically are made up of a cascade of interlinked feedforward loops. Such networks can produce sequential gene activation, as in expression of early and late puff genes in the fruit fly in response to transient edeysone signaling, or steroid-induced cyclic uterine growth. The behavior of individual motifs in isolation needs to be understood first in order to see how they perform in larger “integrated” circuits. The task of informed dose-response modeling is likely to involve determining the circuit structure of the system under study and then focusing on key parts of the circuit affected by chemicals. This talk was followed by an exercise on modeling feedforward loops and developmental cascades.

7.7 Eukaryote cell cycle checkpoint control

The third day began with a talk by Dr Zhang on the network motifs underlying the eukaryotic cell cycle and the mechanisms by which these motifs enable checkpoint control in the cell cycle. The various phases of the cell cycle and the molecular components of the circuit that regulates progression through these phases were explained. The following issues were addressed in the talk:

- The organization of molecular circuits of the cell cycle.
- The drivers of the cell cycle engine underlying the $G1 \rightarrow S$, $G2 \rightarrow M$, and $M \rightarrow G1$ transitions.
- The molecular basis of the irreversibility of these transitions.
- How checkpoint control is enforced under conditions not favorable for proliferation, leading to cell cycle delay or arrest.

A simplified generic model of the eukaryotic cell cycle circuit was described, dividing the cycle into $G1$ and $S-G2-M$ phases (Tyson et al., 2002). This simplified circuit contains multiple network motifs: mutual inhibition (double negative feedback), feedforward, and negative feedback. Mutual inhibition between the molecules cyclin B and Cdh1 generates bistability in this circuit, with $G1$ and $S-G2-M$ as the two stable steady states. Transition from the $G1$ to the $S-G2-M$ phase (flipping the bistable switch) is cell growth (mass)-driven. An additional negative feedback loop mediated by the molecule cdc20 destabilizes the $S-G2-M$ phase, and the resulting oscillatory behavior drives the $S-G2-M$ to $G1$ transition, thus completing the cell cycle. $G1$ checkpoint control in response to DNA damage is enabled by activation of Cdh1, which increases the threshold cell mass required for the $G1$ to $S-G2-M$ transition. This delays the cell cycle and, in the case of excessive DNA damage, can lead to cell cycle arrest in the $G1$ phase. $S-G2-M$ checkpoint control is implemented by inhibition of cdc20 in response to incomplete DNA replication, which blocks the negative feedback loop and the oscillation required for transition to $G1$. This disruption leads to cell-cycle arrest in the $S-G2-M$ phase. The model was demonstrated in the hands-on exercise that followed.

A more detailed model with $G1$, $S-G2$ and $M$ phases as three distinct steady states was described with the example of the fission yeast cell cycle, along with the mechanisms underlying transitions between the phases and checkpoint control. The talk ended with an outline of steps towards implementing a mammalian cell cycle model.

7.8 Stochasticity in gene expression and its implication for dose response, including case studies

Dr Bhattacharya, the next speaker, discussed the topic of stochasticity (noise) in gene expression and its implications for cellular dose-response. The main topics addressed were: i) intrinsic and extrinsic sources of noise in gene expression and mechanisms of noise regulation, ii) dose-response of stochastic bistable switches, and iii) Gillespie’s stochastic simulation algorithm. Depending on the context, stochasticity in gene expression can have both detrimental effects – by creating an unstable, fluctuating intracellular environment; and benefits – for example by allowing an isogenic population of cells to acquire a selective advantage in adapting to different environmental conditions without the need for genetic mutations. Stochasticity also enables differential fate choices in cells making a binary decision, e.g., differentiation vs. proliferation, and it plays a prominent role in physiological pattern formation, e.g., in the compound eye of flies.

Extrinsic noise arises from fluctuations in the levels of different cellular components involved in gene expression, e.g., transcription factors, RNA polymerase, or the protein degradation machinery. Intrinsic noise originates from the innately probabilistic nature of biochemical reactions, which require the collision of reactant molecules in the appropriate orientation. Intrinsic noise is affected by the “finite number effect” – the fewer the number of reacting molecules, the higher the noise; and the relative rates of transcription and translation – higher transcription and lower translation rates produce lower noise levels. Noise also can be attenuated by negative feedback, e.g., through negative auto-regulation of gene expression by transcription factors.

Stochastic fluctuation in protein levels can obscure the true threshold of a bistable switch, causing differential switching among individual cells. This would give rise to a graded dose-response at the cell population level, even though individual cells respond in a switch-like, all-or-none manner. The effect of noise on a bistable switch, and its role in increasing the genetic diversity of a cell population, were illustrated with the example of the vegetative-to-competent state transition in the bacterium *B. subtilis* (Maamar et al., 2007).

The lecture ended with a brief description of Gillespie’s stochastic simulation algorithm, which enables modeling of noise in biochemical reactions, along with a demonstration of the BioNetS stochastic modeling program based on this algorithm (Adalsteinsson et al., 2004).
Proportional negative feedback control and non-linear dose response

In this exercise on the second day of the workshop, Dr Zhang took participants through a model of negative feedback circuits that underlie homeostatic responses to cellular stress. An exercise on proportional feedback control was used to demonstrate how various network motifs embedded in the circuit can enhance local gains and thus the overall loop gain to determine the shape of the dose-response curve.

A bistable gene auto-regulation model

In this exercise led by Dr Bhattacharya, the participants were shown how to model a generic gene auto-regulation system, explore its bistable behavior, and model continuous versus pulse inputs to the system. The use of Berkeley Madonna® to generate trajectories on the phase plane and nullclines to interpret steady states was demonstrated, along with an exercise on graphically deriving the bifurcation diagram from the steady states by varying the input signal to the system.

Creating feedforward loops and developmental cascade

Dr Bhattacharya described ODE models for coherent and incoherent feedforward loops and demonstrated how various biological functions such as the filtering of input noise and pulse generation can be produced by these circuits through appropriate parameter choices. A model of a developmental
cascade combining multiple feedforward loops also was outlined as a representation of ligand-induced changes in the expression of various genes.

**Modeling a simple cell cycle circuit and checkpoint control**
In this exercise Dr Zhang explained how to model the various network motifs that make up the circuit underlying the eukaryotic cell cycle and generate oscillatory behavior to regulate transitions through the phases of the cell cycle. Enhancements to the model to represent checkpoint control in response to DNA damage at the G1 and S-G2-M phases also were described to give participants an overview of computational approaches to understand cell cycle dynamics.

**9 The program wrapped up**

Dr Andersen wrapped up the proceedings with a talk entitled “Computational biology and dose response modeling and risk assessment: where to from here?” He emphasized the role of computational models of pharmacokinetics and cellular signaling pathways, together with targeted *in vitro* assays, for understanding dose-response behavior in chemical risk assessment. The various network motifs that were described and modeled throughout the workshop will be crucial in this process of arriving at a mechanistic understanding of dose-response. A list of various software tools available for biological modeling also was provided to the participants⁴.

**10 Feedback analysis and conclusions**

The workshop drew 59 participants (Fig. 1) representing academia, industry, and research organizations from across India. The participants represented ten Federal States and one Union Territory of India (Fig. 2A,B), and included students, teachers, scientists, and members of animal welfare organizations (Fig. 3).

Participants’ feedback was collected from a questionnaire to evaluate the outcome of the workshop. The questionnaire consisted of 40 items divided into ten domains, ranging from technical expertise to learning experience to hands-on training to hospitality rendered. The domains were titled as overall workshop assessment; workshop hand-outs; workshop conduct; talks on systems biology; technical acumen of trainers; hands-on training; logistics; outcome evaluation; improvements for future workshops; and overall impressions, comments and

Fig. 2A: Participants’ territorial distribution

Fig. 2B: Distribution of participants according to territory

⁴ http://sbml.org/SBML_Software_Guide
been able to present in person. Nevertheless, participants corroborated the format of the training and were very pleased to be trained by experts in the field.

The verdict was commendable for the “hands-on training” domain as well. This domain had three items: a score of 4.23 was obtained for the item “hands-on training was title oriented”; and 4.16 for “hands-on training gave sufficient practice and feedback.” The third item, “duration of hands on training”, was measured by “sufficient” and “not sufficient” choices. 41% of the participants indicated that the time spans were not sufficient. The organizers were gratified by participants’ self-analysis of their knowledge, skills, and confidence with respect to combining laboratory exercises with the use of systems biology tools “before” and “after” the workshop. In this domain each item was graded as poor, fair, good, or excellent. 59% of the participants estimated that they had “fair” knowledge of the systems biology concept and methodology before attending the workshop. However after the workshop, 64% of them claimed a “good” understanding of the concept, and 90% of the participants agreed that the workshop advanced their skills.

Thus, it was clear from the feedback evaluation that the workshop was a considerable success, even with the limitations of long hours required to cover a broad range of material in three days, and the in-person presence of only a single instructor. The participants were very appreciative of the intellectual input and technical expertise of the instructors, and of participating in the first-ever workshop on dose-response modeling in the field of toxicity testing in India.

**References**


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