



t⁴ Workshop Report*

A Framework Program for the Teaching of Alternative Methods (Replacement, Reduction, Refinement) to Animal Experimentation

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Summary

Development of improved communication and education strategies is important to make alternatives to the use of animals, and the broad range of applications of the 3Rs concept better known and understood by different audiences. For this purpose, the Center for Alternatives to Animal Testing in Europe (CAAT-Europe) together with the Transatlantic Think Tank for Toxicology (t⁴) hosted a three-day workshop on “Teaching Alternative Methods to Animal Experimentation”.

A compilation of the recommendations by a group of international specialists in the field is summarized in this report. Initially, the workshop participants identified the different audience groups to be addressed and also the communication media that may be used. The main outcome of the workshop was a framework for a comprehensive educational program. The modular structure of the teaching program presented here allows adaptation to different audiences with their specific needs; different time schedules can be easily accommodated on this basis. The topics cover the 3Rs principle, basic research, toxicological applications, method development and validation, regulatory aspects, case studies and ethical aspects of 3Rs approaches. This expert consortium agreed to generating teaching materials covering all modules and providing them in an open access online repository.

Keywords: 3Rs, education, teaching, toxicology

1 Introduction

Background

The field of alternative methods to animal experimentation has been growing and diversifying during the last years, and it has reached out to many other disciplines. In this context,

the term “alternative methods” is no longer precisely defining (Hartung et al., 2009). We therefore use the expression “3Rs methods” to refer to all approaches aiming at replacement, reduction or refinement of animal experiments. These include for instance integrated testing strategies, pathway-based omics, high-throughput and high-content testing, modeling and

*a report of t⁴ – the transatlantic think tank for toxicology, a collaboration of the toxicologically oriented chairs in Baltimore, Konstanz and Utrecht sponsored by the Doerenkamp-Zbinden Foundation

**Box 1**

For the introduction of modules and for assignment of exercise tasks, case studies were considered a helpful teaching tool. Some examples from different fields are given below.

Examples for case studies:

Case study:	Background and lesson to be learnt:
Recombinant antibodies	The TeGenero trial showed that extensive safety testing in multiple animal species did not ensure human safety (TG1412). <i>In vitro</i> models of a “cytokine storm” based on human cells are claimed to have higher predictively (Stebbing et al., 2007).
Eye irritation	Different <i>in vitro</i> models have been developed and validated in the last years to replace the traditional Draize test in rabbits. Validation studies of the alternatives suggested that the animal model is badly reproducible, and therefore makes any correlative efforts with alternative methods difficult. A combination of several alternative test methods within a (tiered) testing strategy may be able to replace the <i>in vivo</i> Draize test, and several validation studies of different <i>in vitro</i> methods were undertaken or are on-going, e.g., organotypic <i>in vitro</i> assays (isolated eyes), cytotoxicity and cell-function based assays (e.g., red blood cell lysis), reconstructed human tissue (RhT)-based test methods, and quasi- <i>in vivo</i> methods such as the hen egg chorioallantoic membrane test.
Thalidomide	Thalidomide, quite safe in adults, caused serious birth defects worldwide in the late 1950s and early 1960s. After this incident, the requirement of preclinical reproductive toxicity testing of drugs in Europe (EMA) and the US (FDA) started. Additionally, species differences became strongly evident: the compound is hardly toxic to mice and rats, but only to New Zealand white rabbit.
Production of antibodies	Ascites and Freund’s adjuvant were considered indispensable by all scientific experts not so long ago → now many substitutes exist. <i>In vivo</i> generation of monoclonal antibodies was thought to be unavoidable until very recently → now, pure <i>in vitro</i> selection (phage display) and cell culture production are emerging.
Disease models (strategies)	Non-mammalian models and induced pluripotent stem cells from patients with monogenetic and diseases have been used successfully for research and for drug discovery.
Marine biotoxins	The mouse bioassay for marine biotoxin contaminations of seafood can now be replaced by polygenic chemical analytical methods for most of the toxins for which standards are available. The development of functional 3Rs methods in this area is ongoing.
Pyrogen tests	Classical pyrogen testing uses large numbers of rabbits. The clotting reaction of <i>Limulus</i> haemolymph was the first alternative assay for Gram-negative pyrogens. The new generations of pyrogen tests based on the reaction of human immune cells (whole blood) covers the broad range of microbial and synthetic pyrogens relevant to human reactivity.
Pregnancy tests (<i>Xenopus</i>)	Pregnancy tests based on urine injection into frogs and observation of their egg production was state of the art until the 1960s. Antibody-based <i>in vitro</i> methods made such assays completely redundant.
Species differences	Mouse livers are more prone to cancer (e.g., by peroxisome proliferators), than human or other species’ livers; different types of tumors are often found in rodents and man; different brain size and development speed, e.g., postnatal day 5 in rat would still be in pregnancy in humans; different metabolites (e.g., citalopram is toxic to dogs but not to man); failure of stroke, Alzheimer’s and Parkinson’s Disease drugs in man that were successful in rodents; body size affects biokinetics and oxidative stress; different species may differ in (metabolic) enzymes, e.g., mice and rats synthesize vitamin C, humans and guinea pigs do not.
Infections	The case of virulence studies of infectious bacteria shows that each model has its own advantages and disadvantages. There is no golden standard, and quite surprisingly, the <i>Dictyostelium discoideum</i> amoebae model (Lima et al., 2011) has a high predictivity for distinction of clinically virulent strains from non-virulent strains.
Bad experimental design	Numerous examples can be found to illustrate the importance of experimental design and documentation. The ARRIVE guidelines give an example for minimal documentation requirements of animal experiments. Often, the attempt to save animals results in underpowered studies that then need to be repeated with more animals.
Aspirin	Acetylsalicylic acid is an irritant and potential toxicant and tumor promotor in animal models but is used safely in man.
Phototoxicity	The 3T3 NRU phototoxicity test was the first accepted alternative method in the OECD guidelines (TG 432). The 3T3 Neutral Red Uptake Phototoxicity Test, an <i>in vitro</i> assay with the Balb/c 3T3 mouse fibroblast cell line, is based on the comparison of cytotoxicity of a toxic substance in the presence or absence of UVA light. Cytotoxicity is evaluated as a concentration dependent reduction of the uptake of the vital dye Neutral Red.

other *in silico* approaches, and systems biology, as far as they are aligned with the 3Rs principles.

Because of their human relevance, and after extensive validation, several 3Rs methods in the area of toxicology have been adopted in regulatory guidance documents. The continuing progress in the field of *in vitro* methods, e.g., high-throughput and high-content imaging methods and advanced knowledge in the fields of systems biology, protein interactions and gene expression patterns (omics), have opened up new prospects, especially in the field of toxicology, to investigate pathway disturbances and human relevant targets. More generally, 3Rs methods are becoming an essential element in the whole field of biomedical research, from its most fundamental aspects to its daily applications. Today 3Rs methods represent a multidisciplinary scientific area comprising animal science, basic biology, test development, pharmacology, toxicology, regulations and regulatory practices, as well as ethics and behavioral sciences.

According to European Directive 2011/63/EU⁸, all personnel working with experimental animals should be educated to be competent to work with animals. In many countries, courses have been established or are in the process of being established, to meet this requirement. Also other persons not directly involved in animal experiments but having responsibility in one way or another for animal experiments, should be educated and regularly updated on progress being made with respect to the 3Rs in order to maximize their implementation. These persons can be company managers, regulators and journalists *inter alia*.

Because of the complexity of this most diverse field it is mandatory that experts share their knowledge to develop comprehensive teaching programs.

Workshop goals

This workshop, held in Konstanz, Germany, was sponsored by the Doerenkamp-Zbinden-Foundation (DZF) to gather experts in teaching of different aspects of 3Rs methods. The objective was to provide a forum for the exchange of knowledge, definition of the *status quo* and design of a common strategy for improved knowledge dissemination to specific audiences in the field. Moreover, target groups and their specific educational needs were to be identified. A clear understanding of advantages and limitations of 3Rs approaches and animal models should be transmitted to all target groups (Box 1). The definition of lecture modules reflecting the state of the art of the different disciplines of 3Rs methods and 3Rs knowledge was the major task.

2 Results overview

Overall teaching goals

The aim of the dissemination of knowledge on 3Rs methods is to raise consciousness for the scientific soundness of the 3Rs methodology (Fig. 1). A better understanding of the ethical as well as the economic aspects of 3Rs methods will provide a better judgment basis for weighing different approach options.

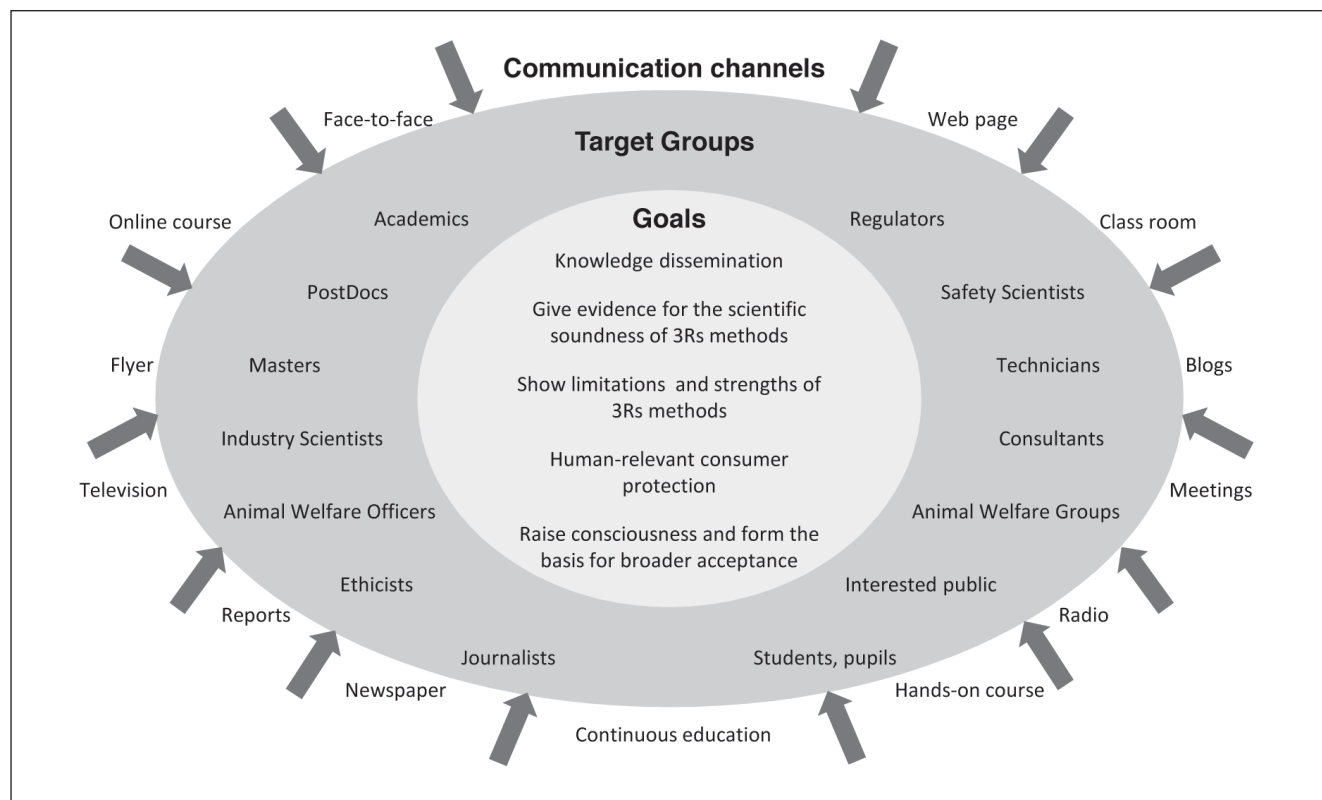


Fig. 1: Overview of the goals, target groups and communication channels

⁸ http://ec.europa.eu/environment/chemicals/lab_animals/pdf/sec_2010_1107.pdf



Finally, an introduction to the new technological approaches in this rapidly developing field of biomedical research will foster an appreciation of the multidisciplinary and progressive nature of the field.

Target groups

Teaching 3Rs methods means covering the various multi-disciplinary aspects of this broad field for different audiences at the appropriate level of detail. The target audiences were classified into five overlapping general groups (Fig. 1):

- a) Graduate scientists, e.g., PostDocs, PhDs, MScs, animal welfare officers
- b) Regulators
- c) Industry scientists, e.g., license holders, principal investigators, ethical committee members (IACUC and IRB), toxicologists (safety testing), pharmacologists, chemists, lab-technicians, regulatory officers, managers
- d) Interested and educated public, e.g., journalists, animal welfare groups
- e) Undergraduate students, pupils

As 3Rs methods also represent an ethical codex that is independent of culture, legislation and religion, the groups targeted by the 3Rs educational approach should span all relevant societal segments. The aim is to counter prejudice against 3Rs approaches by providing evidence of the scientific relevance and validity of 3Rs methods.

Dissemination channels

As the target audience groups have different information requirements, well-established face-to-face teaching in classrooms and hands-on teaching in laboratories but also digital on-line courses, newspaper articles, TV and radio presentations, as well as blogs, meetings and expert reports were determined as worthwhile channels for communication and education (Fig. 1).

Strategy of advertisement

As “alternative methods” was considered an unclear term in a non-specialist context, it was suggested to announce courses under one of the following titles, as appropriate to the target audience: “Life science research in the 21st century”, “Modern approaches in the life sciences”, “New methods in toxicology”, “Theoretical base and methodical superstructure in modern life sciences”, “The revolution of *in vitro* methods in life sciences”.

Such strategic advertisement will draw attention to the topicality and complexity of 3Rs methodology and emphasize the dynamics of this rapidly developing scientific field.

3 Modular structure of the educational program

The complexity of the diverse 3Rs approaches together with the broad range of application fields and different demands of different audiences make the modular concept of the 3Rs education approach inevitable.

The lectures on education worked out by the expert panel will provide an integrated structure, which can be organized as modules addressing different aspects of 3Rs methods. The depth of

detailed content of each module can be adjusted to the requirements regarding the educational and scientific background and interests of the different audiences. This modular education system also allows providing each module as a stand-alone lecture on the professional level.

This modular system (Fig. 2) allows combining theoretical with practical (hands-on) aspects ranging in time span from a few hours to several days. The modules can be complemented with case stories, incorporation of study assignments, and hands-on work.

4 Overview of teaching module content

The following sections reflect the theoretical modules of the framework program worked out during the workshop (Fig. 2).

4.1 Animals in science and society

This module can be regarded as a general introduction to the course.

Animals are used by humans for a variety of purposes. While the use of animals as companion animals in general is perceived positively by society, other contexts of use, e.g., in the food industry, for entertainment purposes, as work animals and as laboratory animals, are seen more ambivalently. Understanding the different societal perceptions of the use and role of animals in science and society helps understand the origins of one’s perceptions and emotional responses and to develop an attitude towards (laboratory) animals that is based on respect and their intrinsic value.

The debate on animal experimentation covering the biomedical benefits, advantages and disadvantages of animal models, basics of animal husbandry, animal laboratory sciences, animal behavior and stress responses should be covered here. Also, the interaction between science and society and the role of societal concerns regarding animal experimentation should be addressed (Kretlow et al., 2010).

The above mentioned aspects can be integrated to offer a framework for ethical considerations on the use of animals; the basic principles of ethics with the relevant decision-making tools, e.g., “Balance of Value against Costs” should be discussed (Festing and Wilkinson, 2007).

This module will generate awareness for identification of moral values from different point of views. For instance, what is the moral value of harming a volunteer in a clinical trial? What if he has signed a consent form? If the benefit is the same, can we take an animal for the same test instead of a person, though the animal cannot understand and sign any consent forms and he is not part of the trial by free will?

4.2 Drivers for change towards 3Rs methods

There are several major drivers for change towards 3Rs methods: scientific developments, societal changes, and economic aspects.

Scientific developments

The scientific limitations and problems with respect to extrapolation of results from animal models to the human situation will be discussed and described along with a variety of examples.

Fig. 2: Overview of the modules of the education courses

Bold letters mark the modules covering different aspects of 3Rs methods, subchapters refer to the content of the teaching modules.

1. Animals in Science and Society Debate on animal experimentation Science and society Societal concerns Animals in environment Ethical consideration in animal use Ethical reasoning and approaches “Balance of value” approaches	6. Alternatives to animals in safety and quality testing II Food Drugs Pesticides Cosmetics Chemicals Biologics	11. Extrapolation and correlation Species differences <i>In vivo</i> → <i>in vitro</i> / <i>in vitro</i> → <i>in vivo</i> In homo approaches Use of biomarkers for risk assessment and safety/quality assurance
2. Drivers for the change towards 3Rs methods Societal change Legal situation, regulations Scientific limitations of animal models New technologies	7. Quality assurance and 3Rs methods Vaccines Food (Shellfish) Endotoxins	12. Study and experimental design Test strategies using multiple tests Test and study quality assurance Definitions of test systems, acceptance criteria, controls Requirement for statistics, data presentation Assay parameters: sensitivity, specificity, accuracy, robustness, potency and efficacy
3. The 3R concept 3R History Reduction Refinement Replacement	8. Teaching and 3Rs Manikins E-learning Computer models Human models	13. Novel approaches HTS, -omics, Image analyses, HCS Tissue engineering and physiology Tox-21c, POT identification Noninvasive strategies
4. Regulatory testing, validation and applicability domains Regulations concerning safety risks Regulations concerning quality assurance Validation of alternative methods Concept of applicability domains	9. Model endpoints Pharmacokinetic info Cytotoxicity, growth, RNA expression, differentiation Function and behavior	14. Disease investigation: non-mammalian models and <i>in vitro</i> methods Transgenic animals Human tissues Disease modeling
5. Alternatives to animals in safety and quality testing I Principles of risk assessment Validated 3Rs (e.g., skin, eye) Emerging 3Rs (e.g., sensitization) Future challenges (e.g., chronic exposure)	10. Bioinformatics and <i>in silico</i> approaches Data mining Algorithms for Integrated test strategies Modeling and simulation QSARs and “read across”	

Also, the technological innovations such as high-content image analyzers, lab-on-chip and high-throughput approaches *inter alia* that now allow us to address scientific questions that cannot be adequately addressed with *in vivo* methods, e.g., toxicological effects of compound mixtures, will be discussed as drivers towards 3Rs methods.

Societal changes

The milestones in societal changes and the associated revisions of regulations regarding the use of animals for research and testing purposes, i.e., introduction of animal tests, national animal protection law, multinational animal protection law, and differences in international animal protection laws as well as the introduction of international test guidelines for approved *in vitro* methodologies by the Organisation for Economic Co-operation and Development (OECD) and the International Conference on Harmonization shall be discussed. These developments have led to the current “No, unless... Principle.”

Economic aspects

Increasingly, economic considerations such as duration (time to market), through-put (as an obstacle for frontloading toxicology in the drug development process) and costs for large-scale

programs like REACH are becoming important drivers. In addition, the potential consequences of wrong business decisions, e.g., made based on extrapolation of animal data to humans, need to be stressed.

4.3 The 3Rs concept

In 1959 Russell and Burch published the important and now classic book, “The Principles of Humane Experimental Technique.” In this book they defined humane science with the concept of the 3Rs – replacement, reduction and refinement. Replacement is the concept of using *in vitro* and other non-whole animal techniques to address a scientific question. Reduction expresses the concept of using the lowest number of animals without compromising scientific soundness and addresses the issue of experimental design. Refinement refers to using techniques and approaches that eliminate or decrease pain and distress experienced by the experimental animals.

An equally important contribution of Russell and Burch is their deduction that good science and animal welfare go hand in hand. This concept is now more generally accepted and is a driving force for appropriate experimental design.

Today the ideas from “The Principles of Humane Experimental Technique” have been incorporated into national and interna-



tional regulations . They are endorsed by organizations around the world, and have a vast economic impact .

Russell and Burch changed the debate on the use of animals from a philosophical matter to a scientific issue. By introducing the principles of refinement, reduction and replacement (3Rs) as goals and guidelines for the work with animal subjects they put a high value on the integrity of living beings in scientific practice.

4.4 Regulatory testing, validation and applicability domains

Most regulatory authorities today rely on well-established animal-based models and test systems to assess the safety and efficacy of consumer products and chemicals, e.g., industrial chemicals, pesticides, cosmetics and pharmaceuticals. Animal testing is performed to determine whether and to what extent exposure to these compounds poses a risk to humans or to the environment.

Regulatory testing

The extent of regulatory testing will be increasing in the coming years as various national and international programs seek to obtain increasing amounts of toxicity information in the face of increasing public concern about the safety of chemicals. For example, large-scale programs for industrial chemicals are the REACH legislation (Registration, Evaluation, and Authorization and Restriction of Chemicals) in Europe and a possible TSCA (Toxic Substance Control Act) reauthorization under discussion in the US (Locke and Myers, 2011). Programs of this type could require huge numbers of animals if current guidance for testing is followed, however, language either exists (REACH Directive), or could be added (TSCA reauthorization), to support the use of non-animal methods wherever possible. Other examples for animal use intensive programs are the US endocrine disruptor screening program and its possible European counterpart. At this moment it is not clear, whether also the increasing concern about nanoparticle toxicity will lead to major testing demands (Silbergeld et al., 2011).

Validation

Before non-animal methods are incorporated into the regulatory standards of most countries they must be “validated” – the process of demonstrating that their use provides equivalent protection to that obtained from animal tests . In order to show that alternative methods can be at least as sensitive, reproducible, and relevant to predicting human health effects as the standard animal methods, national and supranational institutions, i.e., ICCVAM², ECVAM³, JaCVAM⁴, OECD⁵, and more recently KoCVAM in Korea and BraCVAM in Brazil, have been established for validation of alternative methods. Such institutions also function for the purpose of facilitating the implementation of these methods into regulatory testing, which is governed by national and supranational pharmacopoeias, e.g., the European Pharmacopoeia pub-

lished by EDQM⁶ and the US Pharmacopoeia⁷. One key message is that validation is not a process set in stone, but one which needs to be adaptable to different needs and purposes .

Product and chemical risk assessment today uses non-animal *in vitro* tools more and more routinely. For example, advanced cell culture techniques aiming at reflecting absorption (uptake) and chemical metabolism are often used. To support these strategies, quality assurance programs involving “good practice” guidelines (Good Laboratory Practice, GLP; Good Manufacturing Practice GMP; Good Cell Culture Practice GCCP) have been implemented. Additional guidelines, e.g., the ARRIVE guidelines⁸ on reporting of *in vivo* experiments or guidelines on study design and statistical analysis, ensure that the new toxicology methods will provide consistent and reliable results .

This module aims to inform interested parties (including regulators) about the regulatory *status quo*, the use of 3Rs in risk assessment, ITS (integrated/intelligent testing strategies), the role of validation in the acceptance and implementation process of new models and also about new concepts in the area of validation, e.g., the modular approach to validation (Hartung et al., 2004), WoE (weight of evidence validation) , choice of points of reference , the concept of defining applicability domains for validated assays or the application of principles of “evidence-based medicine” .

Applicability domains

Not every chemical can be tested in every test; some types of chemicals are known to result in false positive or false negative test results. The concept of applicability domains is adapted from *in silico* approaches, and defines chemical classes and/or ranges of test method endpoints for which the model makes reliable predictions. The concept of applicability domains describes the fact that an alternative method may be proven valid only for a distinct range of compounds, e.g., the cytosensor microphysiometer test method is considered to be scientifically valid to identify mild ocular irritants only for water-soluble detergents . This new concept of applicability domains allows designing a battery of alternative test systems or a tiered testing strategy covering larger parts of the chemical universe. The applicability domain may also be broadened later by testing a broader range of substances and providing additional evidence of valid results.

4.5 Alternative approaches to animals in safety and quality testing I

3Rs testing strategies represent an opportunity not only to spare animals and limit their suffering, but also to develop human cell and tissue based test systems that more accurately predict adverse effects in humans. At the same time many of these new test systems should be amenable to use in high-throughput modes, which will allow the evaluation of a much larger number of compounds than the time-consuming traditional animal tests. Finally, since

² <http://iccvam.niehs.nih.gov/>

³ <http://ecvam.jrc.it/>

⁴ <http://jacvam.jp/>

⁵ <http://www.oecd.org/>

⁶ www.edqm.eu

⁷ <http://www.usp.org/>

⁸ <http://www.nc3rs.org.uk/page.asp?id=1357>

most *in vitro* tests are easier to interpret than a whole organism, it should be possible to identify directly the cellular mechanisms underlying the toxic effects by these strategies.

This is in line with the current shift in paradigm, from relying on apical endpoints in *in vivo* animal studies to a more basic understanding of adverse effects or Pathways of Toxicology (PoT) in a systems-biology approach (NRC, 2007; . With the maturation of mechanistic high-throughput approaches 3Rs methods have opened to new concepts in risk assessment, moving from animal-relevant *in vivo* methods to human-relevant multi-endpoint assays . The concept has been recently expanded to systematic mapping of pathways of toxicity (PoT), forming the human toxome .

Currently there are validated 3Rs methods, e.g., for skin and eye irritation or for phototoxic compounds, which could in part already be converted to automated high-throughput technologies. Moreover, there are also 3Rs methods in the pipeline, which are very promising, e.g., for evaluation of skin sensitizing compounds.

Future challenges that have to be met in the field of 3Rs methods include addressing the chronic exposure effects of substances. In this regard, reproductive toxicity, repeated dose toxicity, toxicokinetics and carcinogenicity are the fields, where non-animal approaches have to be optimized and validated (Adler et al., 2011; Hartung et al., 2011).

The various approaches should be discussed in the module, which will include pathway of toxicity (PoT) based approaches and Integrated Testing Strategies (ITS), which are necessary, for example, to provide the detailed toxicological information that is required by the European REACH program (Rovida and Hartung, 2009). This module should discuss the principal advantages and limitations of the different approaches. A method critical approach should be applied not only to animal testing but also to its alternatives. *In silico* approaches, which can form an important part of any ITS, are discussed in Section 4.10.

4.6 Alternative approaches to animals in safety and quality testing II

3Rs approaches to animal use in safety testing and quality assurance are to be dealt with in correspondence with different application areas, e.g., food, drugs, pesticides, cosmetics, chemicals, biologics as well as pyrogenic or toxic contaminations. Here different application areas have different demands, different thresholds and thus their own concepts for safety testing.

- **Food:** Food safety problems are often related to microbial contamination, biotoxins (e.g. from algae in shellfish), allergens and chemical contaminations as well as food additives and contact materials.
- **Drugs:** Though there are many animal tests in pharmaceutical industry, the use of *in vitro* methods in pharmacology is also extensive, e.g., for high-throughput screening, for mechanistic studies, for metabolite profiling (P450 from liver microsomes), for genotoxicity (microsomes from arochlor-treated animals) and for cytotoxicity testing.
- **Pesticides:** The US EPA defines pesticides as substances (also mixtures) repelling, controlling, preventing or destroying living organisms regarded as “pests”, i.e., herbicides,

insecticides, rodenticides, avicides, nematocides, antimicrobials, pheromones and genetically engineered toxins. Here alternative skin and eye irritation tests are promising replacement methods. Reduction and refinement alternatives such as tiered acute toxicity tests, the local lymphnode assay and the extended one-generation study for reproductive toxicity, play an enormous role due to the use of up to 6,000 animals per substance. Alternatives for ecotoxicological tests include the zebrafish egg test and the threshold approach for acute fish toxicity testing.

- **Cosmetics:** Relevant for the field of cosmetics there are significant achievements regarding validation and acceptance of alternative methods, e.g., for skin corrosion, phototoxicity, skin absorption, skin penetration, skin irritation, eye irritation, genotoxicity, mutagenicity and in part acute toxicity. On the other hand there are other alternative methods relevant for testing of cosmetics, which have to be optimized and validated: a) skin sensitization, b) toxicokinetics (especially for *in vitro* to *in vivo* extrapolation), c) carcinogenicity testing, especially the completion of the cell transformation assay validation and novel mutagenicity tests due to the high false-positive rate of the existing ones, d) for reproductive toxicity, which is not mimicked completely *in vitro* by the battery originating from the ReProTect project and e) for repeated dose toxicity *in vitro*, which cannot be modeled even with complex *in vitro* systems and thus requires novel concepts such as PoT-based high-throughput testing and systems toxicology (Adler et al., 2011; Hartung et al., 2011).
- **Chemicals:** Notably, there are over 70 million chemicals, which have been synthesized to date. It is estimated that today there are about 100,000 synthetic chemicals consumers in Europe and US are exposed to, but there are safety assessments only for 5% of these substances. If the possible synergistic effects of mixtures or the various formulations of a given substance in nanoparticles should also be determined, there is a need for a tremendous amount of testing in the area of chemical safety .
- **Biologicals:** Biologicals (medicinal preparations made from living organisms and their products, including serums, vaccines, antigens, antitoxins, etc.) are often produced in bacteria or human cells and therefore a variety of special problems is associated with them. Special ways to detect culture contaminants have to be included in each production step and the problem of batch-to-batch variation has to be addressed carefully. Each batch might be unique and has to be tested extensively for safety, quality and efficacy. The regulation and legislation for biologicals is rather complex and in some cases they fall under different laws. Major progress has been made for the safety and efficacy testing of some vaccines and the determination of pyrogenic contaminations (see next section). Another, most prominent, recent example is the testing of Botox, the second most used cosmetic in 2001 (according to the International Society of Aesthetic Plastic Surgeons), which so far required titration of the potency of each batch in a mouse LD₅₀ acute toxicity test, and is therefore one of the biggest animal consumers worldwide. It is regulated as a drug for relatively rare clinical conditions (though this might



change as FDA approved Botox in 2010 for migraine), but more than 90% of all produced Botox is currently used for cosmetic purposes. Noteworthy, the first alternative test has recently (June 2011) been approved by FDA for Allergan, a major Botox producer.

4.7 Quality assurance and 3Rs methods

Quality assurance, safety control and animal testing are traditionally closely linked.

This module will deal with the difficulties and also success stories of exemplary tests on vaccines, marine food and endotoxin contamination (Box 2).

Vaccines

The US National Center for Immunization and Respiratory Diseases states: “For each birth cohort vaccinated with seven vaccines, direct health care costs are reduced by \$ 9.9 billion, 33,000 lives are saved, and 14 million cases of disease are prevented”. Safety and potency of vaccines have to be proven extensively for every batch of the traditionally produced vaccines (in contrast to those produced by gene technology) in animal experiments associated with a significant level of pain and distress. Some examples of recent achievements in the quality control of toxoid vaccines are the replacement of challenge procedures by serological methods, the reduction of numbers of animals required by changing from multi-dose to single dose testing, and developments in the area of *in vitro* models and physicochemical techniques (Hendriksen, 2006).

Marine food

Because food safety is a matter of sensible importance, there are lower detection levels for food contaminants, strict standards, quality controls and monitoring procedures for food, which lead

to a relatively high number of food safety alerts in marine products. The rigorous safety attempts in this field costs in Europe 400,000 lives of laboratory mice each year alone for the mouse bio-assay for accumulated algae toxins in shellfish. An LC-MS/MS replacement for the mouse bioassay for the detection of Diarrhetic Shellfish Poisoning marine biotoxins was recently approved in Europe⁹.

Endotoxin contamination

Pyrogenic (fever-inducing) contamination in pharmaceuticals is a serious health problem. This contamination can be due to microbial (Gram-negatives, Gram-positives, fungi) organisms and their fragments as well as caused by particulate organic and inorganic matter. The Rabbit pyrogen test consumes more than 100,000 rabbits per year in Europe, and reflects the human reactivity only partially. This still high animal use continues despite the fact that to a large part it was replaced by the non-validated Limulus amoebocyte lysate (LAL or Bacterial Endotoxin Test, BET) test. The LAL detects only pyrogens derived from Gram-negative bacteria. The human whole blood assay and some other monocyte activation assays (MAT) were adapted recently, after validation for detection of pyrogens in parenteral drugs. This test represents a functional and physiological assay that has been added to the European Pharmacopoeia as a replacement for the *in vivo* assay.

4.8 Teaching and 3Rs

In 2008, 1.7% (approximately 200,000) of the animals used for experimental and scientific purposes in the EU were used for education and training (Sixth Report on the Statistics on the Number of Animals used for Experimental and other Scientific Purposes in the Member States of the European Union SEC(2010) 1107¹⁰). Animals euthanized before scientific proce-

Box 2

For course discussions it may be helpful to present examples for advantages and limitations of 3Rs models as below:

1. Success of Replacement

Monoclonal antibodies (ascites); Calcitonin batch control (Novartis); Phototoxicity (OECD guidelines); Pyrogen Test; Botox batch control; Goldorfen (*Leuciscus idus*) test for water purity in Germany (DIN 38412 L31); Skin corrosion; Skin irritation, Eye irritation, Genotoxicity

2. Additional, or faster, or cheaper information compared to traditional approaches

Cell culture for pharmacological screening; LLNA for sensitization; New LD₅₀ test approaches; Ecotoxicology acute toxicity testing (daphnia, fish, algae); Non-invasive imaging; Vaccine potency testing

3. Failure of animal approaches

Thalidomide; Delayed organophosphate-induced neuropathy in the rat; Biologicals testing (humanized antibodies, therapeutically used cytokines); Neurodegenerative disease modeling

4. Species non-correlation

MPTP in rat vs. mouse; vomiting response in rodents, Biologicals and immunotherapeutics testing

5. Animals still required

Repeat dose toxicity, carcinogenicity of drugs, behavioral pharmacology, unknown marine biotoxins, biocompatibility of medical devices, cardiovascular regulation

⁹ <http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=OJ:L:2011:006:0003:0006:EN:PDF>

¹⁰ <http://eur-lex.europa.eu/LexUriServ/LexUriServ.do?uri=COM:2010:0511:REV1:EN:PDF>



dures are not considered in these statistics; still, they are used in large numbers for dissection and anatomy classes. Live animals are used, e.g., to teach experimental procedures and laboratory skills for graduate students in disciplines like anatomy, physiology, biochemistry, pharmacology, psychology, parasitology, surgery and toxicology .

In recent years many non-harmful alternatives have been made available for teaching purposes :

- (3D-) models, mannequins and mechanistic simulators
- Film and interactive video
- Computer simulation and virtual reality
- Non-invasive self-experimentation and human studies
- Plant experiments
- Observational and field studies
- Materials from slaughterhouses and fisheries
- Permanently preserved specimens
- *In vitro* studies in cell lines
- Animal cadavers from an animal-friendly and ethical source such as from animals euthanized for medical reasons
- Supervised clinical experience

Teaching with non-animal educational models and the outcome of the learning objectives have been evaluated scientifically, though definitive studies are rare. In the most cases animal-free teaching attained similar or better results . Lack of this knowledge and reluctance of some teachers are still major hurdles for the introduction of 3Rs methods in education (Schmidt et al., 2011). The role of animals and new animal-free methods should be discussed as well as approaches that limit animal use in skills training.

4.9 Model endpoints

Particularly in the field of toxicological risk assessment, information concerning human safety carried out in animal models is limited by the need to extrapolate data from the animal to the human system . There is a need to develop endpoints for 3Rs methods mirroring toxic effects in man. Moreover, for the improvement of the predictive properties of 3Rs methods, testing of a large number of compounds with these methods will be required. Since the molecular targets of toxicity are not known for new substances, a battery of endpoints, such as receptor and enzyme targets, must be combined in the assessment procedure. How primary cell reactions, such as those of glia (Falsig et al., 2004a,b, 2006), can be related to *in vivo* data (Lund, 2006), and then on this basis translated to cell lines (Henn et al., 2009) will explain this concept using a recent example.

This education module will provide strategies to identify relevant endpoints for the evaluation of defined problems with 3Rs methods. It will be necessary to consider, among others, kinetic background information, involving data on cytotoxicity, growth, RNA expression, and differentiation, and function-behavior relationships.

4.10 Bioinformatics and *in silico* approaches

A number of *in silico* tools that contribute to replacing and refining animal testing have entered toxicology (rule-based models, (quantitative) structure activity relationships, virtual organs, kinetic modeling, systems biology, etc.) . The data analysis procedures (DAP) for these methodologies often require calculations

and statistical analysis of the results *in silico*. Statistical evaluation of data or methods can lead to reducing group sizes or abandoning tests shown to be irrelevant.

Many 3Rs methods create computational needs: New 3Rs approaches often need tools for analyzing large amounts of data. Such data usually result from multi-endpoint approaches (omics, image analysis technologies), or high-throughput testing (HTS). Stand-alone “non-testing” alternative methods include (quantitative) structure activity relationships ((Q)SAR), rule-based systems, read-across, etc. The field of computational toxicology also includes modeling and simulation, e.g., physiology-based pharmacokinetic modeling (PBPK) or receptor binding models. All of these aspects have major *in silico* components.

Various approaches and tools are frequently involved in the analysis of data created with 3Rs methods, e.g., data mining, integrated test strategies (ITS) and computational modeling . The educational module concerning bioinformatics and *in silico* methods will inform and train interested parties in handling, analyzing, and interpreting data and information collected with these approaches.

4.11 Extrapolation and correlation

Data collected with 3Rs methods are still subjected to comparative studies with *in vivo* data to prove their validity or in proof of principle studies. Here the concept and application of the *in vitro-in vivo* correlation (IVIVC) draws the attention of pharmaceutical and chemical industry, but also of academia and regulatory players. However, species differences in uptake, absorption, metabolism, kinetics and excretion pose major obstacles for interpretation of IVIVC evaluation, which can sometimes be overcome if the modeling and the parameterization are human-based.

The most relevant systems are *in homo* approaches, such as microdosage application in development and optimization of formulation of therapeutic agent.

This module will teach IVIVC and differences of species with regard to biokinetics and toxicodynamics. Assessment of the value of *in homo* approaches as well as their explanatory power and limitations for different sectors also will be addressed. The involvement of biomarkers for risk assessment, safety and quality assurance using IVIVC will be included in this module.

4.12 Study and experimental design

The quality of study and experimental design is essential for collecting relevant data; generation of a series of experiments based on phenotypically minor variations to refine previous work is a negative example of scientific study and experimental design.

In case of animal studies the choice of species will be discussed as well the role of inbred and outbred animals with respect to reducing animal numbers and increasing scientific validity.

The expert panel recommended for this module to provide definitions for test systems, mediate scientific and regulatory acceptance criteria and define the necessary strategy for collecting adequate controls. Also the statistical background shall be taught to ensure that the requirements, e.g., sufficient replicates for the appropriate statistical analysis, are met. Also the assay param-



eters sensitivity, specificity, accuracy, robustness, potency, efficacy will be introduced using examples of tests and studies (Leist et al., 2010; Zimmer et al., 2011; Stiegler et al., 2011).

4.13 Novel approaches

Robotics, liquid handling devices, sensitive detectors and control software resulting in automated experimentations and data processing enable the creation of millions of data points within a short time. The educational module for novel approaches will provide knowledge on the latest technological developments relevant to the 3Rs field. At present, this module shall provide insight into the field of high-throughput systems (HTS), -omics (proteomics, transcriptomics, metabolomics), image analysis with high-content systems, tissue engineering and physiology, and discuss strategies for identification of pathways of toxicity (PoT).

In this module also current groundbreaking concepts will be addressed, e.g., the idea of Tox-21c (NRC, 2007): this report illuminates how the explanatory power of toxicological risk assessment can be maximized by employing high-throughput *in vitro* screening assays, tests in lower organisms, systems biology, functional genomics and transcriptomics as well as predictive *in silico* approaches.

4.14 Disease investigation: non-mammalian models and *in vitro* methods

It is essential to keep in mind that biomedical research is largely focused on the understanding of human diseases. Despite the interspecies differences reflected *inter alia* in anatomical, biochemical and life span variations, animal models are still widely studied models in biomedical research focusing on human diseases.

The most important tool in the area of disease modeling leading to strong increases in animal use are transgenic animals, which are created and used as disease models. One aim of this course will be to delineate the limitations of and alternatives to the use of mammalian models. First we will cover the use of non-mammalian models. Second we will study the rapid development of *in vitro* methods.

Non-mammalian models

Although transgenic mice (and more recently rats) are heralded as the gold standard of genetic research, many alternative models have been used with extraordinary success, ranging from fish eggs (especially from zebrafish), insects (*Drosophila*), helminthes (*C. elegans*) and plants to unicellular organisms (yeast, dictyostelium). The course will detail a few examples where these alternative models have proven particularly successful, and compare the advantages and limitations of various models.

In vitro technologies

These technologies can be classified into two categories: human and non-human cells *in vitro*. Novel techniques providing human tissues *in vitro* or *ex vivo* already showed a promising potential to reduce animal use and in some cases to replace the animal model. Especially the field of (human) embryonic and induced pluripotent stem cells (iPSC) is receiving large atten-

tion. The generation of cells and tissues with patient-specific background is seen as an important alternative to the use of animal models.

More generally, as our knowledge progresses, more *in vitro* methods are being developed. One of the most striking examples is the development of recombinant antibody technologies which allow selection and production of antibodies *in vitro*.

5 Information and resources

The expert panel of this workshop suggested providing a public online repository with presentations also containing comments of authors regarding the modules discussed in this report. Also a web-based information pool should be created for related and relevant information, e.g., case reports on 3Rs approaches. This should complement more specialized information provided in articles, books and journals.

6 Conclusions and recommendations

The workshop mapped the full bandwidth of 3Rs approaches in biomedical research, toxicology and the life sciences. A variety of target groups could benefit from the offers of such a comprehensive curriculum. Virtual education offers should help to make contents more broadly available. In order to expand the number of teachers, “train-the-trainer” approaches and support by sharing content and materials for education were considered most helpful.

By defining a non-exhaustive list of modules to be considered, a step toward such a curriculum was done. It was recommended to carry out practical trials and document them properly to further define a common curriculum (and its adaption to different areas and target groups). CAAT-Europe is prepared to coordinate these activities, and to offer the different modules to interested parties.

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