REPORT

Workshop On Alternatives To Animal Testing For Food Safety

Sponsored By

ILSI India

International Life Sciences Institute-India

What is ILSI-India?

ILSI-India is a branch of International Life Sciences Institute (ILSI) with headquarters in Washington DC. It works on issue relating to food safety nutrition, toxicology, risk assessment, agriculture sustainability including biotechnology and environment . It works very closely with industry, R&D organizations and government departments, Ministry of Health, Department of Biotechnology, Ministry of Science and Technology, Ministry of Agriculture and Ministry of Food Processing Industries.

ILSI-India carries out its mission through sponsoring workshops, conferences, seminars training programs and research. It also brings out publications and organizes educational programs. ILSI-India activities cover India and South Asian Region.

ILSI is a non-profit, worldwide organization whose mission is to provide science that improves human health and well-being and safeguards the environment. It achieves this mission by fostering collaboration among experts from public and private sectors of society on conducting, gathering, summarizing and disseminating science.

ILSI strategy encourages global action on identifying and then resolving outstanding scientific questions in four thematic areas that capture the core of ILSI's work:

- Food and Water Safety
- Toxicology and Risk Science
- Nutrition, Health and Well-Being
- Sustainable Agriculture and Nutrition Security

ILSI branches include Argentina, Brazil, Europe, India, Japan, Korea, Mesoamerica, Mexico, Middle East, North America, North Andean, South Africa, South Andean, Southeast Asia Region, Taiwan, the Focal Point in China and the ILSI Health and Environmental Sciences Institute. ILSI also accomplishes its work through the ILSI Research Foundation.

ILSI has a special consultative status with Food and Agriculture Organization of the United Nation.

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Workshop On Alternatives To Animal Testing For Food Safety

ILSI-India organized a Workshop on "Alternatives to Animal Testing for Food Safety " on February 27 at India International Centre, New Delhi. The Workshop was chaired by Mr. D H Pai Panandiker, Chairman, ILSI-India, and Co-chaired by Prof. Y K Gupta, Professor and Head, Department of Pharmacology, Dean (Academics), All India Institute of Medical Sciences. The list of participants is given in Appendix 1.

OPENING SESSION

Welcome by Mr. D H Pai Panandiker

Chairman, International Life Sciences Institute-India (ILSI-India)

Mr. Panandiker welcomed the participants and informed them about ILSI-India. He said that ILSI-India was set up 20 years back. It looks after South Asian Region with headquarter in New Delhi. ILSI has 17 branches worldwide and a Research Foundation with Headquarters in Washington DC. ILSI and ILSI-India have the mission to improve public health through science based approaches in the areas of food and water safety, toxicology and risk science, nutrition, health and well-being and sustainable agriculture and nutrition security.

Mr. Panandiker said that while animal testing is widely used as an approach to investigate the safety of food ingredients, novel foods, cosmetic ingredients and pharmaceuticals for human use, scientists have now started looking towards methodologies which do not rely on animal test for safety assessment. In India also testing on animals for cosmetics products and ingredients has been banned. In GM Crops testing on animals is not a regular practice and it is done only if it is necessary. As regards food ingredients animal testing is being practiced.

He said that ILSI-India has set up a Task Force on "Alternatives to Animal Testing with Special Reference to Food Safety" with the leading experts with the following objectives:

- Review the state of art, share views, tackle together this issue and build a consensus.
- Scientifically review / determine what approaches are available or can be developed for food safety, nutrition and efficacy studies.
- Develop a roadmap for development and adoption of non-animal approaches in safety evaluation of food chemicals and ingredients.
- Identify areas where, from a scientific perspective, alternative methods could be applied, but regulatory requirements can only be

fulfilled with animal studies until the Regulators approve validated alternative methods in place.

- Build guidelines on alternatives to animal testing for specific purposes.
- Identify needs for further research to achieve breakthroughs replacing animal studies in the area of nutrition and food safety.
- Disseminate the work and guidelines thoroughly to educate all stakeholders on how alternative strategies can be applied.

He further informed that ILSI has planned to organize a Conference on Alternatives to Animal Testing in 2019 in Asia. The venue is not yet finalized. Members will be informed about further developments.

Observations by Prof. Y K Gupta

Professor and Head, Department of Pharmacology, All India Institute of Medical Sciences

In his introductory remarks Prof. Y K Gupta made the followings observations:

A number of meetings have been held in India over the last 10 years to discuss the issue of alternatives to animal testing. Organization of the Workshop by ILSI-India is timely and appreciated.

Objectives of conducting the safety studies for drugs or food is that whatever comes in contact with humans and environment should be safe. However nothing is 100% safe and therefore, risk-benefit assessment should be done. If the benefits are greater than the risks then a product is considered to be safe. For example: Cancer drugs may cause some side effects, however benefits for the patient is far greater than the risk.

Differences in genetic makeup can be there not only between different species but also within species in different countries and the exposures to a particular ingredients can create toxicity hence it is important that the toxicity studies for establishing safety are conducted/duplicated in India.

List of new foods or new types of foods like GM food / mixed food or new additives / preservatives, as also special packaging materials, should be prepared for analyzing toxicity.

It is also important to undertake studies to find out when food becomes toxic after the date of manufacture or after the package is opened.

Standard methodologies are required for testing safety of foods produced by unorganized sector.

It needs to be identified as to when toxicity testing is required on animal models for GM foods, food additives such as food colors as also

items coming in the food chain such as nano-seeds. Is it possible to do away with animal testing and instead use alternative methods.

It is equally important to identify the critical points where animal testing can be replaced. Such studies can be undertaken by R & D institutions, especially Indian Institute of Toxicology Research. The Task Force should motivate these R & D institutions to intensify such studies.

Once the methodologies have been identified they should be validated and publicized. They must be placed before the regulators for their acceptance.

Leaching from packaging materials is also an area for study. It should be studied as to whether there is any leaching in packaging materials such as PET and if there is leaching whether it is within safe limits.

Bioinformatics can be useful in prediction of toxicity.

Shelf life and the best before use date are other areas of study. In case of drugs purchased by US army, it has been found that even after the expiry date drugs are safe with rare exceptions. This study has been published in British Medical Journal (BMJ). The Government of India is also considering if drugs are safe after the expiry date. Studies can be undertaken on processed foods. In vitro methods must be standardized to evaluate whether a product is safe after the expiry date.

Presentations by Members of the Task Force on Work Initiated in India

1. Dr. B Sesikeran, Former Director, National Institute of Nutrition and Chairman, Review Committee on Genetic Manipulation (RCGM), Department of Biotechnology, GOI

Dr. Sesikeran was not able to join the Workshop but he sent a note expressing his views. These are given below:

Animals have been used as surrogates for establishing efficacy as well as safety of chemicals, drugs and pharmaceuticals as well as food. Even traditional medicines were tested hundreds of years back in many countries by either observing animals when they consume them by their natural instincts or by feeding them intentionally. There have been anecdotal instances of human studies without animal experiments but these were aberrations and best forgotten.

In recent years even animal experiments are being questioned and the use of animals for cosmetic research is largely not approved.

With tremendous advancements in cell biology, tissue culture, in-vitro methods, imaging techniques, molecular mechanistic studies, advanced computer simulations, databases, structural homology

studies and many more techniques it is now possible to avoid animal experiments to establish proof of concept and efficacy and even predictive toxicity.

In drug research the development of more and more protein molecules like monoclonals and human or humanized proteins the random use of animals is no more meaningful since these molecules are so human cell specific that they will not function in animal models, and therefore, cannot predict toxicity either.

In In-vitro systems, the cell line studies, provide the essential information to arrive at effective as well as toxic doses. Toxicity can be derived only if the animals are responsive models. Transgenic animals have been used and can provide reliable data in a small number of animals.

The only area where use of a significant number of animals is still unavoidable is in regulatory toxicology. Since adverse effect of molecules can be seen only in a real life physiological system, and not in isolated cells, validations of in-vitro or ex-vivo methods are a challenge.

In silico methods could possibly replace a whole lot of animal testing since computer simulations can incorporate several variables and almost mimic a living system. These may still have to be validated with parallel animal studies for some time before regulation can accept them as evidence of safety.

In the Department of Biotechnology, over the years, animal testing protocols have been minimized for all recombinant DNA-derived molecules to a bare minimum and all predictive in-vitro processes have been given priority as adequate evidence of efficacy and safety. Phase 1 and 2 clinical trials have very often shown no concurrence with the observations obtained from animal experiments for large molecules.

Lower animals like invertebrates or lower vertebrates have now been validated and are being used and several larger animals have been spared. A combination of several such test methods will be able to totally replace animal use in experiments in the near future.

2. Dr. A B Pant, Principal Scientist, CSIR-IITR (Indian Institute of Toxicology Research) on behalf of Prof. Alok Dhawan, Director CSIR –IITR

Regulatory decisions are based on scientific data in various areas such as food additives/ contaminants, labeling, novel food processing, standardization, responsibility (liability), food irradiation, genetic modifications etc., with the objective to protect consumers' health. Alternative methods to animal testing are being developed from the point of view of animal welfare and ethics but ultimately they have to be adopted by the regulatory regime.

With respect to use of animal testing scientific and technical advancements are underway for replacement, reduction, refinement, relevance, rapidity, reproducibility, reduced cost, and responsibility. It needs to be underlined that new alternative methods are being developed but not at the expense of human safety.

India received the status of full adherence to member country in the OECD system for the "Mutual Acceptance of Data (MAD) in the Assessment of Chemicals", on April 2011. This means that data generated in signatory countries are not replicated and animal testing is reduced.

IITR has adopted multi-institutional umbrella projects on development and validation of in-vitro/alternate models for the safety/toxicity assessment of drugs/cosmetics/new chemical entities in 2002. This project is still in progress.

IITR has developed in-vitro model systems including the following:

1. In Silico Modeling (1000 compounds)
2. In Vitro Models (100compounds)
3. Alternate Models (10 compounds)
4. In Vivo Models (1 compound)

IITR has also standardized for the country the following OECD-approved in-vitro methods:

Skin absorption: In-vitro skin absorption assay (TG428)
Skin irritation and skin corrosion: Transcutaneous electrical resistance test (TG430), Membrane Barrier Test Method for Skin Corrosion (TG435), Reconstructed Human Epidermis Test for skin corrosion (TG431, TG439), Skin Sensitisation (TG442D)
In vitro phototoxicity: 3T3 NRU Phototoxicity Test (TG432),
Skin sensitization: Local Lymph Node Assay: DA (TG442A), Local Lymph Node Assay: BrdU-ELISA (TG442B); In Chemico Skin Sensitisation (TG442C);

Receptor assays: Transcriptional activation in HeLa-9903 line to detect estrogen receptor agonists and antagonists (TG455, TG457); H295R steroidogenesis assay (TG456)

In vitro ocular corrosives and severe irritants test: Fluorescein leakage test in MDCK tubular epithelial cells (TG460)

Genetic Toxicology

- Bacterial Reverse Mutation Test (TG471)
- In vitro Mammalian Chromosome Aberration Test (TG473)
- In vitro Mammalian Cell Gene Mutation Test (TG476)
- In vitro Mammalian Cell Micronucleus Test (TG487)
- Reconstructed Human Cornea-like Epithelium for the Detection of Chemicals Not Requiring Classification and Labeling for Eye Irritation or Serious Eye Damage (TG491)
- In vitro Thymidine Kinase Mutation Test (TG492)

Ecotoxicology Studies

- Freshwater Alga and Cyanobacteria: Growth Inhibition Test (TG201)
- Daphnia sp.: Acute Immobilization Test (TG202); Reproduction Test (TG211)
- Fish: Acute toxicity (TG203); prolonged toxicity (TG204), early-life stage toxicity (TG210); acute and early embryonic stage short term toxicity (TG212, TG236); Juvenile Growth Test (TG215); Reproduction Assay (TG229); Estrogenic/androgenic activity, and aromatase inhibition assay (TG230); Sexual Development Test (TG234)
- Earthworm: Acute Toxicity (TG207); Reproduction Test (TG222)
- Terrestrial Plants: Growth Test (TG208); Vegetative Vigour Test (TG227)
- Honeybees: Acute Oral Toxicity (TG213); Acute Contact Toxicity (TG214); Acute Larval Toxicity (TG237)
- Lemna sp.: Growth Inhibition Test (TG221)

- IITR has developed model for Cytotoxicity, Ocular Irritancy Testing, Epidermal Skin Cytotoxicity Model and Alternative to animal model for in vivo male reproductive toxicity assessment.
- The following alternatives model are under development in IITR:

  Genotoxicity: (MN, CA, Comet assay)
  Immunotoxicity: (Stem cell derived dendritic cells)
  Neurotoxicity: (Stem cell derived neuronal and glial cells)
  Phototoxicity: (NIH3T3, L929 cells)
  Xeno estrogenicity: (MCF-7)
  Cytotoxicity: (L929, LAL test)
  Acute/sub-chronic/ chronic toxicity/ Reproductive Toxicity: (C. elegans, Earthworm, Zebra Fish)

IITR has published 300 research publications, review articles, book chapters, etc. in reputed journals.

IITR has also undertaken international collaborative projects with: UK-IERI, Major and Standard, British Council, UK, European Union (FP-7): NanoLinen and NanoValid, Indo-Brazil, etc.

Data generated by IITR have been adopted by the regulatory bodies such as ECVAM, and EU document on safety of nanomaterials.

IITR has undertaken study on endocrine disruptor activity of nutraceuticals. The following work in progress:

YES/YAS assays (Yeast strains expressing human ER/ AR & reporter gene)
Transcriptional activation in HeLa9903 line (OECDTG455)
Steroidogenesis in H295R line (OECDTG456)
Uterotrophic-rat (OECDTG440)
Hershberger-rat (OECDTG441)
Pubertal female-rat, Pubertal male-rat
Fish short-term reproduction

Dr. Pant also informed about alternatives to animal testing models being developed in other countries. For example: US FDA is currently testing whether livers-on-a-chip-miniature models of human organs engineered to mimic biological functions can reliably model human responses to food-based and food-borne illnesses. The purpose is to test toxicity of food additives and screen organ specific responses of food-borne pathogens. Dr. Pant informed about the international agencies that validate alternatives. These are:

European Centre for the Validation of Alternative Methods (ECVAM)- Established in 1992
Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM)- USA 1997
Japanese Centre for Validation of Alternative Methods (JaCVAM)- in 2005
Korean Centre for Validation of Alternative Methods (KoCVAM)- 2009
International Cooperation on Alternative Test Methods- in April 2009 (an agreement signed between Europe ,USA, Canada, Japan and Korea
Canadian Centre for Alternatives to Animal Methods- 2012

Dr. Pant felt that there is a need for setting up an "Indian Centre for Validation of Alternative Methods" (IndCVAM). The objectives would be to institutionalize the alternative methods to build cooperative relationships with both domestic and international organizations and to review and validate proposed alternatives. IndCVAM will be intending to respond to the global trends by introducing/promoting alternative test methods developed by Indian organizations.

The IndCVAM activities could include the following:

Providing policy support to the development and acceptance of alternative test methods that replace animal testing
Executing validation and peer review of new and revised alternative test methods and proposing related guidelines
Building cooperation with both domestic and international organizations and participating in international collaborative studies

Providing education and training and information regarding alternative test methods

Join the membership of the International Cooperation on Alternative Test Methods (ICATM). Other members of ICATM are ICCVAM, EURL ECVAM, JaCVAM, KoCVAM, and Health Canada.

3. Dr. B. Dinesh Kumar, Scientist F & HoD, Drug Toxicology Division, National Institute of Nutrition (NIN)

NIN also works as a Contract Research Organization and it has the mandate for pre-clinical evaluation: Efficacy Evaluations, Safety Evaluation (Regulatory Toxicology), Pharmacokinetics and Toxicokinetic studies and Pharmacopeial Requirements.

At NIN's Laboratory of Animal Science the following principles are adopted:

Replacement - relative replacement, direct replacement , and indirect replacement

Reduction - internal lab system, non-animal methods, proper statistical design, use of inbred animals and rehabilitation of animals

Refinement- minimize potential distress, enhance animal wellbeing and initially use in-vitro models

There has been paradigm shift in drugs safety evaluation. The conventional methods use isolated receptor/enzymes, intact cells, isolated organs and intact animals with more emphasis on use of intact animals and least on isolated receptor/ enzymes. In the modern system there is more emphasis in silico / in-vitro approaches followed by isolated receptor/enzymes wherein isolated organs and use of intact animal are minimized.

There are barriers in replacement of alternative methods. These include validation barrier, scientific barrier, legislative barrier, developmental barrier, psychological barrier, fear of litigation barrier and regulatory barrier.

The in-vitro toxicology provides information on mechanism of action of a drug and early indicator of the potential for some kinds of toxic effects, allowing a decision to terminate or to proceed further on drug development. In vitro methods are widely used for screening and ranking chemicals, getting a platform for animal studies for physiological actions, studying cell, tissue, or target-specific effects and improving subsequent study design.

The major advantages of in vitro toxicology is that it is faster than in-vivo study and less expensive to run and less predictive of toxicity in intact organisms.

The following in-vitro methods have been developed/ are being developed to study toxicity: cytotoxicity, protein binding, CYP

inhibition / induction, membrane permeability, immunotoxicity, metabolism and kinetics.

A number of alternative test methods have been validated in Europe by European Union Reference Laboratory for European Centre for Alternatives to Animal Testing (EURL-ECVAM) in the toxicity areas of : carcinogenicity screening, skin sensitization screening and need for hazard, acute oral toxicity screening, toxicokinetics screening, eye irritation screening and hazard, endocrine disruption screening. Appendix – 2.

The OECD has adopted a number of alternative methods for regulatory purposes in the areas of toxicity: skin corrosion replacement hazard, skin irritation replacement hazard, eye irritation reduction, need for ITS, carcinogenicity screening, genotoxicity and endocrine disruption screening. Appendix – 3.

NIN has adopted the following tests / models in the area of alternatives to animal testing.

in-silico evaluation (Advanced Centre for Bioinformatics).
LAL test (Endotoxin)
Cytotoxicity (in-vitro– Ames mutagencity test) - [Comet assay (bone marrow]
Caco2 cell model for assessing the nutrient bioavailability: DBT Networking Center for Bioavailability Screening.
Cell lines for screening – splenocyte, immuno-modulation activity, anti-cancer drugs (MCF7)
Interaction studies of T-cells and adipose tissues in obesity
Ex-vivo systems.

The DBT networking center has adopted in-vitro Caco-2 cell model. The center possesses all the regulatory machinery for absorption of nutrients and drugs.

A study on effect of various dietary components on zinc bioavailability in Caco-2 cells has shown that the results correlate with human and animal studies.

Table 2. Comparison of Zinc Absorption Ratios in Caco-2 Cells (Present Study) and in Humans (Literature) from High and Low Phytate Meal

			ratio	
study	phosphorus (mg/g)	absorption (%)	phosphorus (B/A)	absorption (B/A)
whole soy meal (A)[a]	4.0	22.8	0.0075	1.51
dephytinized soy meal (B)	0.03	34.6		
whole bread (A)[b]	2.85	8.2	0.292	1.60
white bread (B)	0.833	13.2		
HEWF (A)[c]	5.81	0.62	0.187	1.46
LEWF (B)	1.09	0.91		

[a] From ref *35*. [b] From ref *36*. [c] Present study; HEWF, high-extraction wheat flour; LEWF, low-extraction wheat flour.

Source: From Presentation by Dr. Dinesh Kumar

Only 43% of data generated in rodent toxicology predicts human toxicities. Non-rodent toxicity matches 63% of human toxicities.

NIN has a center for advanced research for pre-clinical toxicology with the following divisions: drug toxicology research center, pathology, national centre for laboratory animal sciences, immunology and statistics with well-trained team of scientists with the required specialization.

It is important not only to reduce but replace animal tests and promote in-vitro evaluations for regulatory toxicology. However, each method has to be scientifically validated. It is necessary to build infrastructure for this purpose.

4. Dr. Debabrata Kanungo, Consultant – Medical Toxicology

Using animals as humans substitute to test products for safety began with Greek anatomists.

Around 115.3 million animals are used in experiments annually (conservative estimate).

The move for adopting alternatives to animal testing came from people concerned with animal welfare.

The 3 R's concept being used today can be defined as follows:

Replacement : Refers to the preferred use of non-animal methods over animal methods whenever it is possible to achieve the same scientific goal.

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

Refinement : Refers to methods that alleviate or minimize potential pain, suffering, or distress, and enhance welfare of the animals used.

There are number of national regulations for promoting animal welfare. Given in Appendix - 4.

The international regulations are given in Appendix – 5.

Animal testing is much more expensive than non- animal models. Further, use of non-animal models increases the scope of more compounds being tested.

Alternatives to use of animals in testing include the following:

In vitro (test tube) test methods and models based on human cell and tissue cultures
Computerized patient-drug databases and virtual drug trials
Computer models and simulations
Stem cell and genetic testing methods
Non-invasive imaging techniques such as MRIs and CT Scans.

Micro-dosing (in which humans are given very low quantities of a drug to test the effects on the body on the cellular level, without affecting the whole body system).

The benefits of non-animal testing include the following:

Alternative scientific tests are often more reliable than animal tests.
The use of human tissue in toxicity testing is more accurate than the animal models/tests.
Non-animal tests are more cost-effective, practical, and expedient.
Cruelty-free products are more environment-friendly.

Switzerland and Germany are setting up new centers for alternatives to animal testing.

The European Union has banned use of animal for testing of cosmetics from all perspectives in 2013. This has provided impetus for the development of non-animal alternative tests and has seen the European cosmetics industry – the biggest in the world – grow and innovate.

Several other countries have followed suit and put bans in place.

Legislation is currently under consideration in Australia, Brazil and Canada.

Waiting for change one-country-at-a-time- is slow and can lead to a patchwork of differing laws. Hence harmonization is important.

5. Dr. G. Taru Sharma, Principal Scientist and Head, Director, CAFT, Physiology & Climatology Division, ICAR-IVRI (Deemed University)

Dr. G. Taru Sharma was not able to join the Workshop but she sent a PowerPoint presentation expressing her views. These are given below:

IVRI-ICAR has been working on stem cells as alternatives to animal testing. However, it is important to understand the full spectrum of stem cell actions to propose them as alternatives to animal testing.

There is a need for novel animal models to expand the range of current studies, most of which have been conducted in rodents.

These differences can preclude the ability to reproduce the results of animal-based preclinical studies in human trials.

Major stem cell applications are:

Tissue remodeling and engineering.
Understanding early embryonic development.
Model for in vitro drug and immunity screening.
Producing environment friendly transgenic animals e.g., enviro pig.
Pharmaceuticals: Diabetes, cardiac anomalies, neurodegenerative diseases, and infertility
Conservation of endangered species.

Major challenges facing use of stem cells are: Finding suitable stem cell source, successful isolation and culture of stem cells, successful differentiation into specific cell types and development of an efficient cryopreservation protocol.

The different sources of stem cell are embryonic stem cells, MSCs from bone marrow, MSCs of fetal origin and MSCs from adipose tissue.

The advantages and disadvantages are given in Appendix- 6.

Stem cell-based therapy provides new prospects for regenerative medicine and also opens up the horizon to use them as an animal alternative.

A national repository of stem cells should be set up.

6. Prof. M A Akbarsha, Coordinator-Research, National College

Established in 2009 the Mahatma Gandhi- Doerenkamp Center, Bharathidasan University (MGDC), and went up to 2016, exclusively from a grant from Doerenkamp-Zbinden Foundation, Switzerland, has conducted 75 training workshops on digital alternatives for dissections and physiology experiments across the country; 22 training workshops each on in vitro toxicology; 5 training workshops on handling of 3D RHE models; and one training workshop each on Integrated discrete Multiple Organ Co-culture (IdMOC) and Systems Biology. The Center has also been engaged in original research on alternative modalities.

MGDC is now taken over by UGC-funded National Centre for Alternatives to Animal Experiments (NCAAE).

US National Academy of Sciences brought out "Roadmaps for Toxicity Testing for 21st Century-Tox 21c"- in 2007. These are based on the new principle for safety testing without animals by employing the novel molecular and computational techniques, e.g., the "FDA Predictive Toxicology Roadmap".

In January 2016 ICCVAM launched the "Strategic Roadmap for Establishing New Approaches to Evaluate the Safety of Chemicals and Medical Products in the United States the Lauterenburg Amendment to Toxic Chemicals Act of USA". Among other things this amendment requires that experiments on mammalian model organisms need to be dispensed with.

The US FDA no longer considers validation of test methods as important. Accordingly to it "Rather than validation, an approach we frequently take for biological (and toxicological) models and assays is qualification. Within the stated context of use, qualification is a conclusion that the results of an assessment using the model or assay can be relied on to have a specific interpretation and application in product development and regulatory decision-making."

The new developments in in-vitro models are: multiple organs on a chip and 3 dimensional cells culture models to mimic the conditions of in-vivo. These models can be used for the applications in toxicology, cancer biology, food processing, etc. The MGDC has been pioneering the development of 3D models for in vitro toxicity testing / drug discovery to motivate the toxicology / pharmacology community in India to replace animal models with 3D in vitro models. The principal aim here has been "Make the Alternative Models in India" so as to encourage use of alternative models at affordable cost.

In addition to the different levels of in vitro models, invertebrate alternative model organism can obviate the use of the highly sentient vertebrate models. Even here, lower invertebrate models would carry very many advantages of size, availability, life span, agility, etc.

Hydra, which evolved 540 millions ago, is a fresh water animal with unlimited availability. The whole genome has been sequenced. It has many conserved pathways with humans, it can be used to test toxicity with simple morphological and/biochemical/molecular biological end points. It is a model organism for developmental biology. Hydra culture is inexpensive and requires fewer instrumentation. It can be reared in laboratory in a BOD Incubator. Hydra is used in MGDC for in-vivo, ex-vivo and in-vitro toxicity testing.

It is important to study the effects of migration of nanomaterials in food products, degradation of bio-polymers, fate of nanomaterials in environment and effects of nanomaterials in aquatic organisms as also regulatory compliance. Unintentional/intentional releases of nanomaterials into the aquatic environment inflict serious threat to the benthic organisms and thereby access the higher organisms including man via food web. It is important for food industry to understand that Nano-technology is being used in packaging materials for promoting hygiene and safety as also traceability. Given in Appendix – 7. Hydra has been used to evaluate the toxic effect of bulk as well as nanomaterials. MGDC's recent experiments with hydra showed that nanoparticle can easily penetrate the cell barrier (outer epithelial layer). A newer dimension to nanotoxicology is Nanomaterials with antimicrobial properties. It not only impedes pathogens but also inhibits growth of commensal bacterial species, thereby induce damage to the animal/human health.

7. Dr. R. Thirumurugan, Professor and Coordinator, NCAAE, Bharathidasan University

As a continuum of MGDC, the UGC-funded National Centre for Alternatives to Animal Experiments (NCAAE) Bharathidasan University has the following as objectives:

To establish a comprehensive database of literature on animal alternatives.
To establish a repository of digital and simulation alternatives for dissections and animal experiments and to bring upnewer digital alternatives
To establish. the facilities for and provide training in in vitro alternatives; to establish co-culture facility and 3D culture facility; to bring up new technologies / variants of existing technology.
To establish the facilities for and provide training in in silico alternatives technologies.
To offer academic programs on "alternatives".
To network individuals, institutes and labs engaged in contributing to alternatives; to conduct seminars, symposia, conferences and national and international congresses on alternatives. In this regard, recently we have conducted two national workshops on techniques "Methods in cell culture and in vitro toxicology".
To establish Indian Society forAlternatives to Animal Experiments.

NCAAE facility is involved in the following:

Cell-based assays, in the form of test batteries and/or as part of tiered testing schemes to predict human toxicity endpoints, which will be a key technology in the place of animal testing.
Alleviate the problem of shortage of skilled manpower in the domain of interdisciplinary streams by providing training in in vitro toxicology / pharmacology.
Develop its own models for metabolic disease adopting IdMOC technology.
Develop its own models toculture cells in 3D forms as like in vivo arrangement of the body.
In the education domain, the facility empowers the institutions and faculty with information, knowledge, material and expertise in the digital and simulation alternatives.
Facilitate use of in silico tools to screen the toxicity of chemical substances.
Use of Hydra-a lower model organism- as envisaged by Tox 21- for eco-toxicity testing.

8. Dr. K M Chacko, Director, Shri Ram Institute of Industrial Research

Shri Ram Institute has toxicology division and an animal house which is GLP certified.

Experiments have to be conducted as per Government regulations and protocols. Therefore, alternatives methods can be used only if they are Government approved.

Use of alternative methods will help the industry.

9. Dr. Sanjay K Banerjee, Scientist E, Drug Discovery Research Center, Translational Health Science and Technology Institute (THSTI)

THSTI mostly uses animals for studies.

Neutral Red uptake cytotoxicity assay has been proposed to identify substances not requiring classification as acute oral toxicants under EU regulations. The neutral red uptake assay is a cell viability assay that allows in-vitro quantification of xenobiotic-induced cytotoxicity. This is mainly used for hazard assessment in in-vitro toxicology applications. This procedure is cheaper and more sensitive than other cytotoxicity tests.

In vitro studies have demonstrated the intestinal absorption using CaCo-2 cells. They have shown that toxicokinetic information can be generated based solely on in-vitro data, with the resulting data being in the same order of magnitude as those published for human volunteers. Caco-2 cells are derived from human colon adenocarcinoma. Caco-2 cells as a model, however, has several limitations, such as the long time it takes to form tight junctions, wide variation with passage number, variability between different laboratories and underestimation of paracellular transport of compounds because they have tighter junctions compared to human intestinal enterocytes. Also, Caco-2 cells express low amount of CYP3A enzymes which is a major metabolizing enzyme for many drugs; thus, transfected cell lines expressing high amount of these enzymes have been used to study CYP3A substrates.

Another alternative model is the TC-7 cell line which is a subclone isolated from Caco-2 cells. These cells have similar characteristics as Caco-2 cells in addition to their ability to express CYP3A enzymes.

10. Dr. Vijay Pal Singh, Deputy Director, Risk Assessment, Research & Development Special Projects, Food Safety and Standard Authority of India (FSSAI)

FSSAI has 11 sets of regulations. These are posted on the website.

While developing standards EU regulations, Codex and BIS standards are referred to. No animal testing is conducted.

Quality of research is very important.

In case of proprietary / non-specified product approval, safety studies on humans are accepted.

As regards toxicity studies, at present FSSAI has listed 4 tests, out of which 2 tests are alternative. These tests include: chromosomal aberration tests, reproductive toxicity tests and pre natal development toxicity studies. For allergenicity, adverse effect study in humans is accepted. For determining MRLs, data generated by CIBRC is accepted.

DISCUSSIONS

It is important to identify the tests of foods / ingredients where animal testing can be replaced by alternative methods.

Capacity building is important.

Once an alternative method has been developed it is important to validate this method across multiple validation centers.

Indian Center for Validation of Alternative test Methods (IndCVAM) should be established.

Test methods have to be specified not only under national regulations but also under WTO and SPS for wider acceptability.

FSSAI should prepare guidelines on toxicity testing.

No whole food items require animal testing.

Toxicity testing is required for food ingredients / preservatives and nutraceuticals, flavors, packaging materials, including with nanomaterial, and pesticide residues in food products. If a natural food is produced with more of any component such as red palm oil with more beta carotene than available conventionally then this will require animal testing. Animal testing is required for testing acute toxicity.

Codex guidelines can be checked for the types of toxicity studies which are required in food additives, ingredients.

Codex 2016 guidelines and JECFA toxicological requirements should be studied.

Research on Alternative Methods can be funded by Ministry of Environment, Ministry of Food Processing Industries, Department of Biotechnology and FSKAN of FSSAI.

Next Steps

ILSI-India Task Force on "Alternatives to Animal Testing with Special Reference to Food Safety" should study as to what are the areas in food, food ingredients, contaminants, additives, antibiotic studies where toxicity testing is required in India or internationally and where animal testing is required.

It should also study where animal testing can be replaced by Alternative Methods which are already available. If methods are not available then it needs to be investigated whether Indian scientific institutions can develop methods.

The toxicological requirements under Codex were agreed to be studied by Dr. Banerjee and Dr. Kanungo.

ILSI-India should send a letter to FSSAI requesting them to provide information on the requirements for toxicological testing under Food Safety Act and Rules.

Action should be imitated to set up "Indian Centre for Validation of Alternative Methods". The objectives would be to institutionalize the alternative methods to build cooperative relationships with both domestic and foreign organizations and to review and validate proposed alternatives. The IndCVAM activities could include the following:

- Providing policy support to the development and acceptance of alternative test methods that replace animal testing
- Executing validation and peer review of new and revised alternative test methods and proposing related guidelines
- Building cooperation with both domestic and foreign organizations and participating in international collaborative studies
- Providing education and training andinformation regarding alternative test methods
- Join the membership of the International Cooperation on Alternative Test Methods (ICATM). Other members of ICATM are ICCVAM, EURL ECVAM, JaCVAM, KoCVAM, and Health Canada.

FSSAI can be approached to evolve guidelines for toxicity testing and clarifying areas where use of animal use can be avoided/minimized.

Appendix – 1

Workshop On Alternatives To Animal Testing For Food Safety

February 27, 2018

List of Participants

ILSI-India Task Force Members

- Prof. Mohammad A Akbarsha, Coordinator-Research, National College (Autonomous)
- Dr. Sanjay K Banerjee, Scientist E, Drug Discovery Research Center, Translational Health Science and Technology Institute (THSTI)
- Dr. K M Chacko, Director, Shri Ram Institute of Industrial Research
- Dr. Y. K. Gupta, Professor and Head, Department of Pharmacology, Dean (Academics), All India Institute of Medical Sciences
- Dr. Debabrata Kanungo, Expert (Medical Toxicology and Human Health Risk Assessment), Former Additional Director General, Ministry of Health and Family Welfare, Government of India
- Dr. Rajni Kaul, Scientist G, Indian Council of Medical Research (ICMR)
- Dr. B. Dinesh Kumar, Scientist F & HoD, Drug Toxicology Division, National Institute of Nutrition, Indian Council of Medical Research (ICMR)
- Dr. G. Harish Kumar, Scientist, SERC-Division, Department of Science and Technology, Technology Bhavan
- Mr. D H Pai Panandiker, Chairman, ILSI-India
- Dr. A B Pant, Principal Scientist, CSIR-Indian Institute of Toxicology Research
- Dr. Vijay Pal Singh, Deputy Director, Risk Assessment, Research & Development,
  Special Projects, Food Safety and Standards Authority of India (Ministry of Health & Family Welfare), Government of India
- Ms. Rekha Sinha, Executive Director, ILSI-India
- Dr. R. Thirumurugan, Coordinator, National Centre for Alternatives to Animal Experiments (NCAAE), Bharathidasan University

Invitees

- Mr. Sunil Adsule, Director - Scientific and Regulatory Affairs, Coca-Cola India Pvt. Ltd.
- Dr. Sanjeev Kalia, Seeds Regulatory Manager, Regulatory Science APAC-2, Bayer CropScience Limited
- Mr. Manoj Kumar Rajput, Manager QA, National Dairy Development Board

ILSI-India Secretariat

- Mrs. Premavathi, Manager
- Ms. Ashima Jawa, Assistant Manager
- Ms. Dipti, Secretarial Assistant

Appendix – 2

Status Of Validation Of Alternative Test Methods At EURL ECVAM Since 2010

No.	Toxicity area	Test method description	Validation status
1	Carcinogenicity Screening:	Cell Transformation Assay (CTA) SHE	EURL ECVAM recommendation published in 2011
		Cell Transformation Assay (CTA) Balb/C	EURL ECVAM recommendation published in 2011
		Cell Transformation Assay (CTA) BHAS	ESAC peer review finalized
2	Skin sensitization *Screening and need for ITS for hazard*	KeratinoSens test method	ESAC peer review finalized
		Direct Peptide Reactivity Assay (DPRA)	ESAC peer review finalized
		human Cell Line Activation Test (h-CLAT)	ESAC peer review foreseen to start in 2013
3	Acute oral toxicity *Screening*	3T3 Neutral Red Uptake (NRU) test method	EURL ECVAM draft recommendation to be published in April 2013
4	Toxicokinetics *Screening*	Cytochrome P450 (CYP) induction assay using the human cryopreserved HepaRG® cell line and cryopreserved human hepatocytes	ESAC peer review foreseen to start in 2013
5	Eye irritation *Screening and need for ITS for hazard*	Reconstructed human tissue model (EpiOcular™ EIT)	ESAC peer review foreseen to start in 2013
		Reconstructed human tissue model (SkinEthic™ HCE)	ESAC peer review foreseen to start in 2013
6	Endocrine disruption *Screening*	MELN® estrogen receptor transactivation assay (agonist and antagonist protocols)	ESAC peer review foreseen to start in 2013
		Androgen receptor transactivation assay (agonist and antagonist protocols)	EURL ECVAM validation foreseen to start in 2013
		Androgen receptor transactivation assay (agonist and antagonist protocols)	EURL ECVAM validation foreseen to start in 2013
European Union Reference Laboratory for alternatives to animal testing (EURL-ECVAM)			

Source: Presentation by Dr. B. Dinesh Kumar, Scientist F & HoD, Drug Toxicology Division, NIN (National Institute of Nutrition), Hyderabad

Appendix – 3

Status Of Regulatory (OECD) Acceptance Of Alternative Methods Since 2010

No	Toxicity Area	Test Method Description	Acceptance Status
1	**Skin corrosion** ***Replacement*** ***Hazard***	**Reconstructed human Epidermis test methods (RhE) as included in OECD TG 431/EU TM B.40 bis**	**Accepted in 2004, updated version (subcategorisation, performance standards, inclusion of SkinEthic™ RHE and epiCS®) adopted at WNT in 2013**
		Transcutaneous electrical resistance (TER) test as included in OECD TG 430/EU TM B.40	**Accepted in 2004, updated version (performance standards) adopted at WNT in 2013**
2	**Skin irritation** ***Replacement*** ***Hazard***	**Reconstructed human Epidermis test methods (RhE) as included in OECD TG 439/EU B.46**	**Accepted in 2010, updated version (performance standards, inclusion of LabCyte EPI-model) adopted at WNT in 2013**
3	**Eye irritation** ***Reduction, need for ITS***	**Fluorescein Leakage (FL) test method as included in OECD TG 460**	**Accepted in 2012**
		Bovine Corneal Opacity and Permeability (BCOP) test method as included in OECD TG 437/EU TM B.47	**Accepted in 2009, updated version (positive control, use in a bottom-up approach to identify nonclassified chemicals) adopted at WNT in 2013**
		Isolated Chicken Eye (ICE) test method as included in OECD TG 438/EU TM B.48	**Accepted in 2009, updated version (use in a bottom-up approach to identify non-classified chemicals) adopted at WNT in 2013**
		Cytosensor Microphysiometer (CM) test method	**New draft TG discussed at WNT in 2013 but not yet adopted, pending further clarification on its use in a bottom-up approach**

4	**Carcinogenicity** ***Screening***	**Cell Transformation Assay (CTA) SHE**	**New draft TG discussed at WNT in 2013 but not yet adopted , need for a Guidance Document**
5	**Genotoxicity**	**Existing OECD TGs under revision**	**Draft OECD TG 473 (*in vitro* chromosome aberration assay) and OECD TG 487 (*in vitro* micronucleus test) will be discussed at WNT in 2014**
6	**Endocrine disruption** ***Screening***	**Estrogen receptor transactivation assay (BG1Luc ER TA; agonist and antagonist protocols) as included in OECD TG 457**	**Accepted in 2012**
		Performance-Based Test	**Accepted in 2012**
		Guideline for Stably Transfected Transactivation *In Vitro* Assays to Detect Estrogen Receptor Agonists (OECD TG 455)	

Source: Presentation by Dr. B. Dinesh Kumar, Scientist F & HoD, Drug Toxicology Division, NIN (National Institute of Nutrition), Hyderabad

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Appendix – 4

Laws and Regulations – National

1960	**Prevention of Cruelty to Animals Act 1960, and Amended 1982**
1964	**Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA)**
2001	**Guideline for Care and Use of Animals in Scientific Research (INSA)**
2009	**MCA Amendment - Recommend to use Alternatives to Animal Experiments**
2012	**Animal use Bans in Educational Institutions**
2013	**UGC Guideline for Discontinuation of Dissection and Animal Experiment in Zoology at Graduation Level**

Source: Presentation by Dr. Debabrata Kanungo, Consultant – Medical Toxicology, Faridabad

Appendix – 5

Laws and Regulations – International

1959	Publication of 3 R' s Concept
1981	OECD Council decision – Mutual Acceptance of Data; Center for Alternatives to Animal Testing (CAAT)
1986	EU Directive 86/609 for the protection of animals used for experimentation
1991	Establishment of the ECVAM(European Centre for the Validation of Alternative Methods) - Eurotox
1997	Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM)-A permanent committee of the NIEHS under the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM)
2008 2016	TOX21 EU-ToxRisk – An Integrated European 'Flagship' Programme Driving Mechanism-based Toxicity Testing and Risk Assessment for the 21st century

Source: Presentation by Dr. Debabrata Kanungo, Consultant – Medical Toxicology, Faridabad

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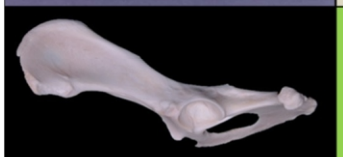
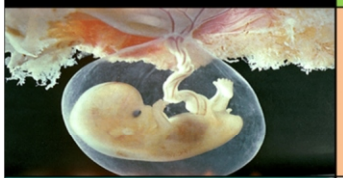
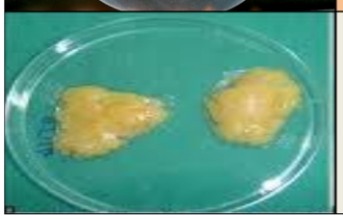
Appendix – 6

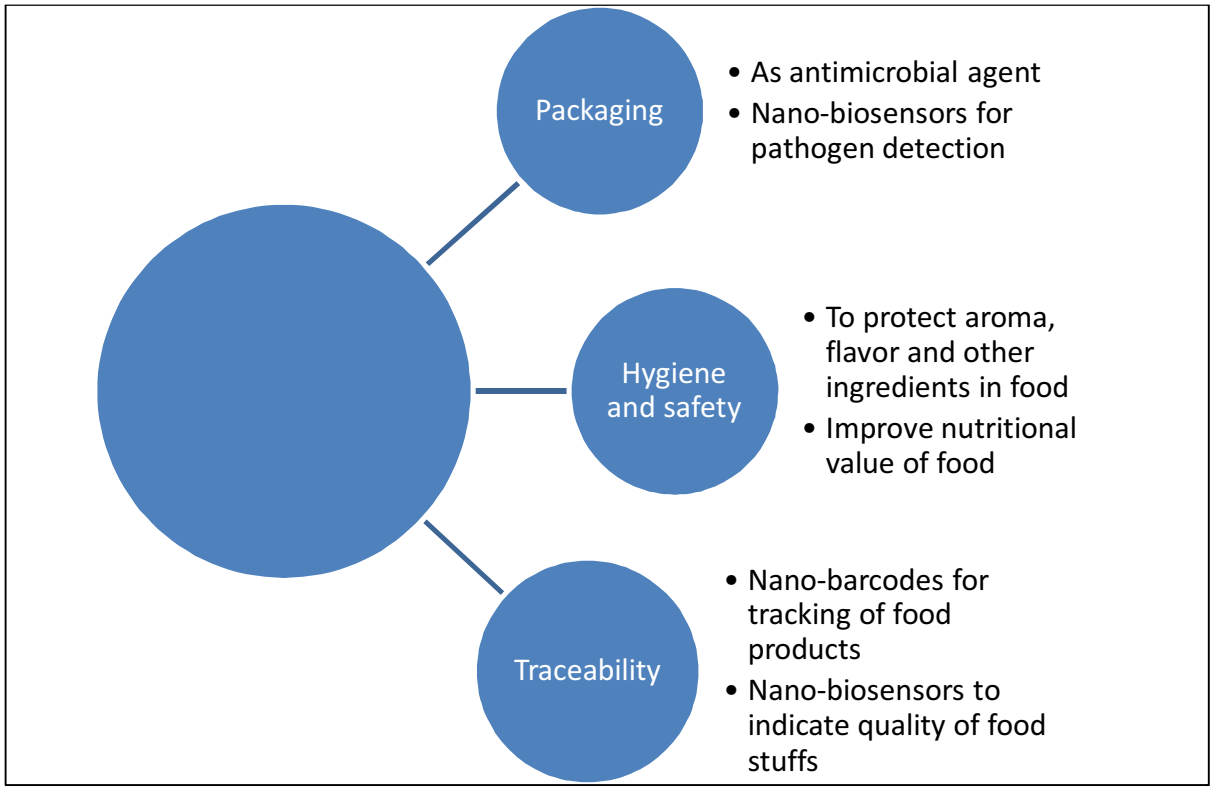
Advantages and Disadvantages of Using Stem Cells

Stem Cell sources		Advantages	Dis-advantages
	Embryonic stem cells	True pluripotentiality	Poorly controllable – teratoma formation when implanted.
	**MSCs from Bone marrow**	Easy to recover. Extensively researched	More limited differentiation potential, Low numbers requiring culture for selection and expansion.
	**MSCs of Fetal origin**	Might have greater multipotentiality, Easy to recover.	Too few numbers in umbilical-cord blood, Few data on cells from umbilical cord itself.
	MSCs from Adipose tissue .	Easy to recover	More donor-site morbidity than for BM, Limited knowledge about these cells compared to those from BM.

Source: Presentation by Dr. G. Taru Sharma, Principal Scientist and Head, Director, CAFT, Physiology & Climatology Division, ICAR-IVRI (Deemed University), Izatnagar

Appendix – 7

Nanotechnology Applications In Food Industry



Source: Presentation by Prof. M A Akbarsha, Coordinator-Research, National College (Autonomous), Tiruchirappalli

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