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Alternatives 2007

Workshop on Alternative Methods *(Reduction, Refinement, Replacement)* to Animal Tests

Programme and Abstracts

12-13 November 2007
Hacettepe Congress Centre
Ankara, Turkey

co-organised by

Hacettepe University
Turkish Pharmacological Society &
European Commission, JRC, IHCP, ECVAM

TÜRK FARMAKOLOJİ DERNEĞİ 1966

40th year

ECVAM

Introduction to ECVAM

The European Centre for the Validation of Alternative Methods (ECVAM) is a unit of the Institute for Health and Consumer Protection of the European Commission's Joint Research Centre (JRC) at Ispra, Italy.

By establishing ECVAM, the European Commission made a significant addition to Europe's commitment to the orderly development, validation and acceptance of replacement alternative methods. In a communication to the Council and the European Parliament in October 1991, the Commission pointed to a requirement in *Directive 86/609/EEC*, on the Approximation of Laws, Regulations and Administrative Provisions of the Member States Regarding the Protection of Animals Used for Experimental and Other Scientific Purposes, that:

> *The Commission and Member Stares should encourage research into the development and validation of alternative techniques, which could provide the same level of information as that obtained in experiments using animals, but which involve fewer animals or which entail less painful procedures, and shall take such other steps as they consider appropriate to encourage research in this field.*

The European Commission established ECVAM to:

- coordinate the validation of alternative test methods at the European Union level;
- act as a focal point for the exchange of information on the development of alternative test methods;
- establish, maintain and manage a database on alternative procedures;
- promote dialogue among legislators, industries, biomedical scientist, consumer organisations and animal welfare groups, with a view to the development, validation and international recognition of alternative test methods; and
- help expand the JRC's role in research.

ECVAM thus seeks to promote the scientific and regulatory acceptance of alternative methods, which are of importance to the biosciences, through research, new test development and validation, and the establishment of specialised databases, with the aim of contributing to the replacement, reduction and refinement of laboratory animal procedures (in accordance with the Three Rs concept of Russell & Burch).

Today ECVAM's main mission as a unit of the Institute for Health and Consumer Protection is:

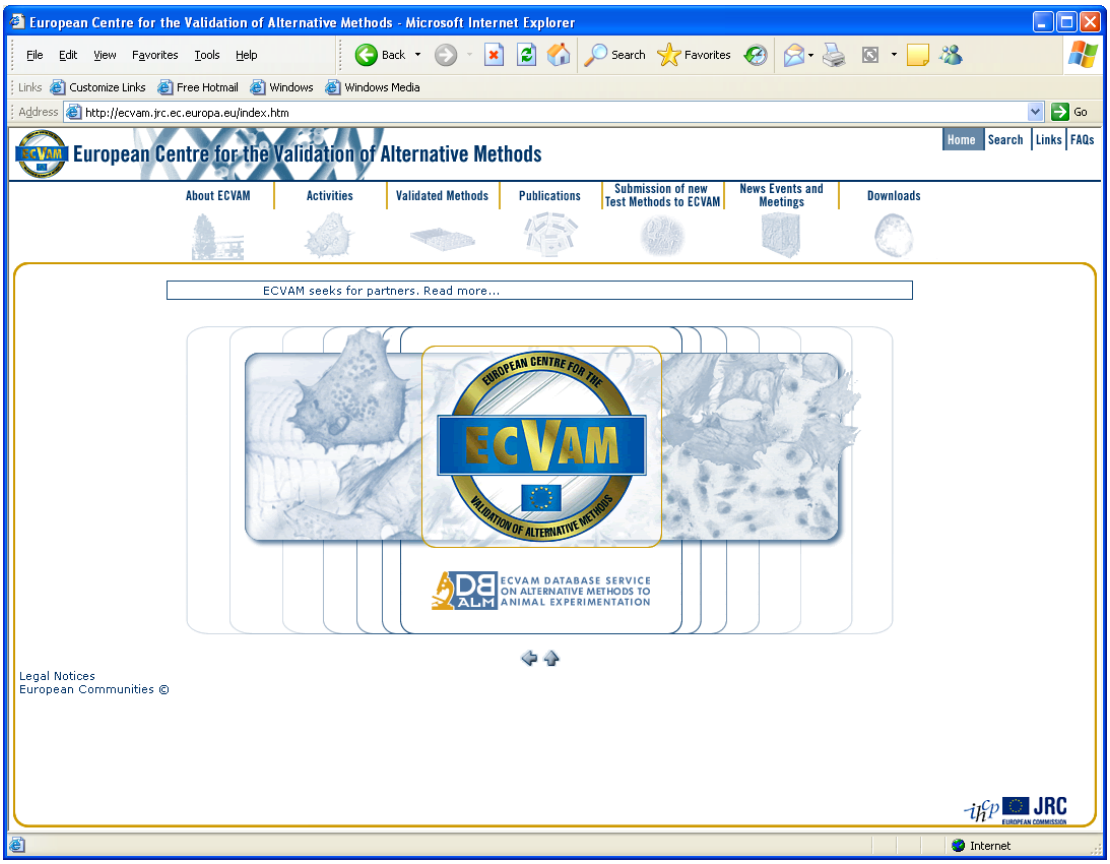
> *to coordinate the independent evaluation of the relevance and reliability of tests for specific purposes, and in particular through prevalidation and validation studies, so that chemicals and products of various kinds, including medicines, vaccines, other biologicals, medical devices, cosmetics, household products and agricultural products, can be manufactured, transported and used more economically and more safely, whilst the current reliance on animal test procedures is progressively reduced.*

Because of its unique nature, ECVAM has its own Scientific Advisory Committee. In addition to representatives of the 27 Member States and other Commission Services, the Commission has appointed to this Committee members selected from nominations made by invitation: the European Cosmetic, Toiletry and Perfumery Association (COLIPA), the European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC), the European Federation of Pharmaceutical Industries Association (EFPIA), the European Society for Toxicology In Vitro (ESTIV), the European Consensus-platform for Alternatives (ecopa), the European Science Foundation (ESF) and EUROGROUP for Animal Welfare, and observers from various organisations: the Organization for Economic Co-operation and Development (OECD), the Interagency Coordinating Committee on the Validation of Alternative Methods (ICCVAM), the National Toxicology Program Interagency Center for the Evaluation of Alternative Toxicological Methods (NICEATM) and the Japanase Centre for Validation of Alternative Methods (JaCVAM).

Since not all of the interested parties could possibly be represented on its Scientific Advisory Committee, ECVAM has also established a wide network of contacts with appropriate individuals and organisations, not only in Europe, but throughout the world.

For further information please visit

ECVAM - http://ecvam.jrc.it
IHCP - http://ihcp.jrc.ec.europa.eu/
JRC - http://ec.europa.eu/dgs/jrc/index.cfm



Hacettepe University

Hacettepe university (HU) is located in two major campuses in Ankara. Sihhiye (Central) Campus in downtown Hacettepe neighbourhood, hence the university owes its name, is also called the “Health Sciences Campus”. Three faculties (Medicine, Pharmacy and Dentistry), six institutes, and five schools as well as the Department of Health Administration of the Faculty of Economics and Administrative Sciences are located in Hacettepe Campus. In addition, there is the Hacettepe Medical Centre with a 1,150 bed adult hospital, the 350 bed Ihsan Dogramaci Children’s Hospital and a 180-bed oncology hospital. Overall, almost 10,000 undergraduate and 1,500 graduate students in allied health sciences continue their studies in Sihhiye Campus, which makes almost 40% of the total HU body. The remaining part is located in Beytepe Campus, 17 km off city center, where Engineering, Economics, Physical & Natural Sciences, Arts & Humanties, Fine Arts and Communications Faculties contiue to serve to a total of more than 20000 undergraduate and graduate students. University has several vocational schools, one state conservatory and many research centers and institutes.

Hacettepe University is a highly praised university in Turkey in terms of international publications. The total number of 1448 indexed publications from HU in 2005 is well above the number of publications from many world universities. Although the total number of publications is increasing, the share of the experimental studies involved animal experimentation follows a steady pattern. Hacettepe University is the foremost institution in terms of clinical trials and other clinical studies of international nature in Turkey. EU-based institutions share more than 10% of the total scientific output of the Hacettepe University.

Hacettepe University Faculty of Medicine has opened an animal breeding facility and an experimental surgery unit back in 1960s. In 2003, the facility was moved to its present location: a new building laid out in a standard 3-aisle plan, with special ventilation/humidity and light-dark cycle controls. Modern husbandry methods were later implemented with the support of State Planning Organisation. The upgrade process consisted of a three-phase plan destined to improve the overall animal welfare and allow breeding of high quality strains. In the first phase physical structure was upgraded with the addition of adequate wash stations, a human resources management was put in rigor, and SOPs for workflow and routine maintenance were developed. The second phase was the procurement of new cage systems, post-operative support units, anaesthesia-euthanasia equipment, routine interventions laboratory, intensive-care units and monitoring equipment. Third phase was the implementation of inbred and outbred husbandry methods, acquisition of certified animals to raise certified colonies of rats and mice. As a result of these improvements, animal-breeding facility won the official breeder certificate from the Ministry of Agriculture in 2007.

Regarding the institutional organization of the research management, Hacettepe University with its research ethics committees maintains a good position compared to other Turkish universities. Members of the Laboratory Animal Breeding Unit participated actively in committees working on adaptation of local legislation to EU acquis in terms of laboratory animal ethics and animal breeding facilities. University

bylaws prevents using laboratory animals without the Ethics Committee approval and all personnel using animals in research should obtain a certificate through training programs.

For further information please visit

HACETTEPE UNIVERSITY- www.hacettepe.edu.tr
ETHICS BOARDS- www.etikkurul.hacettepe.edu.tr

Turkish Pharmacological Association

The Turkish Pharmacological Society was founded in 1966. At the very beginning, the society was nothing but a group of highly motivated academics. Back in 1960s, there were only three departments of pharmacology offering PhD degrees in Turkey. The 1970s had an enormous impact on the transformation of Turkish pharmacology into a publication-driven research discipline. The first international publications in SCI journals came around 1960s. As new universities emerged all over the country their efforts to recruit young graduates for experimental research helped in shaping a new generation of pharmacologists. The number of publications reached a critical threshold 25 years later in the latter half of the 1990s, and the annual output in SCI journals exceeded 200 by the 2000s. In 2005, pharmacology departments in Turkey published 312 articles, of which more than 5 % involved collaboration with EU researchers.
In 1975, the word “Turkish” was added to the title with a government decree and the society gained recognition as the representative of all Turkish pharmacologists. The Turkish Pharmacological society joined the Federation of European Pharmacological Societies (EPHAR) as founding member in 1990. Turkish Pharmacological Society is also member of the International Union of Pharmacolgy (IUPHAR). Today with its 554 active members, it is one of the “larger” societies in Europe.
In the midst of frugal founding years, the first national pharmacology meeting was held, in 1973 owing to personal efforts of a handful pharmacologists. The latest national meeting (19^{th}) was held in Trabzon in 2007. The national meeting also enhances international participation and integration of local pharmacologists into the global community. The 17th, was organized as a joint Turkish-Dutch Pharmacological Societies Meeting and in the past many leading scientists, among them Nobel Laureate Prof. Robert Furtchgott, have given unforgettable lectures and enjoyed the hospitability of their Turkish colleagues.
From the late 1970s on, apart from the national meetings, TPS has established the tradition of having regular symposia and workshops. Winter symposia are held in the memory of late pharmacologists. The main objective of these meetings is to improve scientific and professional face-to-face interaction between the senior and upcoming members of the society. It is rightfully coined as an “initiation ceremony” where post-doc level researchers present their first independent studies as well as their future projects. Spring symposia are “mind opener” type of meetings to give in depth and up to date information on new techniques and instrumentation that pharmacologists need to be aware of for their future work. Those activities serve also to enhance the social atmosphere among the members.
The 554 members of Turkish Pharmacological Society, are mostly medicine or pharmacy graduates. Pharmacologists are mainly members of the academia (more than 90%), however, a growing number is employed in drug industry and in public service sector.
Since 1998 TPS has decided to create task groups to meet the increasing demand from its members whose activity has already became quite diverse. The “Clinical Pharmacology Workgroup” organises workshops and symposia to create awareness among clinicians (and other professionals) on rational drug use, pharmacovigilance

and clinical trials. A more recently formed group is named “Clinical Toxicology Workgroup”. Its activities aim to promote cooperation among pharmacologists and toxicologist working in poison control centers and hospitals, as well as in research laboratories. TPS members are active participants in all administrative decision bodies that regulate drug registration, clinical trials, drug prescription and experimental use of animals in Turkey. These workgroups close the gap between pharmacologists and other members of the medical community and create a cooperation platform.

For further information please visit

TURKISH PHARMACOLOGICAL SOCIETY- www.tfd.org.tr

Programme

12 November 2007

08:00 Registration

09:00 Opening & Welcome of participants
Tunçalp Özgen, President, Hacettepe University, Mehmet Melli, President TPS, Marlies Halder, ECVAM

Session 1 – Ethical & Regulatory Background

09:30 Introduction to the Three Rs concept
Marlies Halder, ECVAM

09:50 Protection of laboratory animals in Turkey
Hakan S. Orer, Hacettepe University

10:20 Coffee break

10:45 The work of ethical review committees in Germany and the United Kingdom – from the point of view of animal welfare
Ursula Sauer, on behalf of Royal Society for the Prevention of Cruelty to Animals, UK)

11:15 Report of an animal welfare officer
Fred Poelma, University Utrecht

11:45 Discussion

Session 2 Validation of alternative methods and their use

12:00 Introduction to ECVAM and its activities
Marlies Halder, ECVAM

12.45 Lunch

14:00-14:30 ECVAM Key Area Systemic toxicity – summary of ongoing activities
Agnieszka Kinsner, ECVAM

14:30-15:00 ECVAM Key Area Topical toxicity – summary of ongoing activities
Chantra Eskes, ECVAM

15:00-15:30 Report of a visiting scientist at ECVAM – The Establishment of an In vitro Method for Pulmonary Toxicity
Yüksel Cetin, ECVAM

15:30 Coffee break

16:00-16:30 ECVAM's activities in the area of in vitro neurotoxicity
Agnieszka Kinsner ECVAM

16:30-17:00 ECVAM Key Area Sensitisation – summary of ongoing activities
Chantra Eskes, ECVAM

17:00 Discussion

17:30 Close of Day 1

18:00 Reception

13 November 2007

Session 3 Alternatives in higher education

08:30 The use of alternatives to animal tests in higher education
David Dewhurst, Edinburgh University, UK

09:00 Introducing alternatives to using animals in teaching in Balkan universities
Zvesdana Z. Kojic, University of Belgrade, Serbia

09:30 Demonstration of programmes
David Dewhurst, Edinburgh University, UK

10:00 Coffee break

Session 4 Good Cell Cultures Practice

10:30 ECVAM's guidance on GCCP
Agnieszka Kinsner, ECVAM

Session 5 Wrap-up of the workshop

11:00 General discussion

12.00 Closure of the workshop and Lunch

Abstracts

Introduction to the Three Rs concept

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What are now know as the Three Rs of Russell and Burch, *replacement, reduction* and *refinement*, have their origins in a proposal made in 1954 by Charles Hume, founder of the Universities Federation for Animal Welfare (UFAW, UK), that UFAW should undertake a scientific study of humane techniques in laboratory experiments. W.M.S. Russell and R.L. Burch were appointed to carry out the work, which led to their book, *The Principles of Humane Experimental Technique* (1959).

They defined *replacement* as “any scientific method employing non-sentient material which may in the history of animal experimentation replace methods which use conscious living vertebrates”, *reduction* as a means of lowering “the number of animals used to obtain information of a given amount and precision”, and *refinement* as any development leading to a “decrease in the incidence or severity of inhumane procedures applied to those animals which have to be used”.

The presentation will give an overview on the progression of the Three Rs concept into national and international legislation and guidance documents and will provide examples on its implementation in the quality control of vaccines, e.g. deletion of no longer relevant tests, use of humane endpoints, single-dose assays instead of multi-dose assays, use of serological methods for potency testing.

Recommended ECVAM workshop report (to be downloaded from http://ecvam.jrc.it):

The Three Rs: The Way Forward – Report and recommendations of ECVAM workshop 11; Balls et al, 1995, ATLA 23, 838-866

Protection of Laboratory Animals in Turkey

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Due to the crucial decision by the Turkish universities to promote international integration, there has been a dramatic turn in the second half of 1990s in research activities that involve laboratory animals in Turkey. The decision to adopt the new “research assessment criteria” required that researchers publish in SCI-listed journals in order to be eligible for academic promotion. This change coincided with the establishment of animal care and use committees in many countries and a committee approval has fast become obligatory to go through the editorial process. In Turkish universities, animal ethics, particularly laboratory animal ethics, had been a “remote” topic that had largely been confined to veterinary schools. It took almost another decade to adopt the Animal Protection Act, the very first law regulating animal use for experimental purposes in the country. With the increase in the number of studies using laboratory animals, universities started to establish their individual laboratory animals ethics committees to meet the criteria set forth by the journal editors.

Hacettepe University was one of the pioneering institutions to review the research proposals in a systematic way. In 1996, an ethics committee on laboratory animals was appointed by the Medical School. Demands from other faculties/schools of the university prompted the University Senate to expand the ruling of this committee to the whole university in 2000. Several other universities have followed suit. Apart from the universities, TÜBITAK (Scientific and Technique Research Council) was one of the few institutions that has taken into account the issue of the laboratory animal use by imposing an ethics committee approval for all grant applications involving animal use. Despite these developments, no initiative was present at the national level to regulate the animal experimentation. However, starting from 2000, Turkish-EU integration efforts have gained momentum and the transformation of the Turkish system to incorporate “Acquis Communautaire” paved the way to the present administrative structure.

In 2004, the Ministry of Agriculture and Rural Works issued a regulation on the breeding and husbandry of laboratory animals, which required all relevant facilities to meet specific technical and operational criteria. In addition, the Ministry of Environment and Forestry promulgated the regulation on Laboratory Animals Ethics Committees in 2006 and established the National Ethics Committee for Animal Experiments.

Present situation:

- All institutions using laboratory animals should appoint an ethics committee to monitor the usage, breeding and husbandry of the laboratory animals.
- The committee should review all of the demands to use laboratory animals, approve experimental protocols in line with the 3R principles.
- The committee should supervise the conditions of the animal facilities and monitor their anesthesia and euthanasia protocols.
- All individuals who wish to perform manipulations on living animals should receive appropriate theoretical and practical training of minimum 80 hours. Training programs should be approved by the Ethics Committee and include alternative methods to animal use.

Local ethics committees should report their activities to the “National Ethics Committee for Animal Experiments” appointed by the Ministry of Environment and Forestry. National committee acts as the highest competent authority to implement policies on animal use and resolve disputed issues.

Since these legal and administrative frameworks have been put forward recently, the implementation and impact of the regulation on scientific research have not yet been evaluated.

Presently, the Ministry of Agriculture and Rural Works has started to perform site visits and award certificates to the institutions, which pass the inspection. The universities are eager to adapt the new regulation, as least at the administrative level. It is expected that the national ethics committee system will become fully operational within a year.

The work of ethical review committees in Germany and the United Kingdom – from the point of view of animal welfare

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In the European Union, the performance of animal experiments is regulated by Directive 86/609/EC on the Protection of Laboratory Animals. Article 7 (2) and (3) of this Directive state that animal experiments shall not be performed if *"another scientifically satisfactory method of obtaining the result sought, not entailing the use of an animal, is reasonably and practicably available"*. Likewise, in a choice between experiments, those *"which use the minimum number of animals, involve animals with the lowest degree of neurophysiological sensitivity, cause the least pain, suffering, distress or lasting harm and which are most likely to provide satisfactory results shall be selected"*. These requirements follow the "principle of the 3Rs" established by the scientists Russell and Burch in 1959. The 3Rs (Replacement, Reduction, Refinement) call for replacing animal tests by test methods with insentient material, or, if this is not possible, for reducing the numbers of animals used to the absolute minimum, and refining husbandry and procedures to reduce any associated pain, suffering, or distress, taking into account the experience of the animals from birth to death.

All EU Member States have implemented Directive 86/609/EC into their respective national legislations. Even though the concrete provisions differ from country to country, most Member States have enacted an authorisation procedure for animal experiments and have included some form of ethical review process therein. In accordance with the German Animal Welfare Act, scientists who want to perform an animal experiment in Germany have to submit a written application for authorisation to the responsible authority. They have to explain the scientific goals they want to pursue and demonstrate that the proposed animal experiment is indispensable in accordance with Article 7 of Directive 86/609/EC, and they are called to classify the severity of the distress expected to be inflicted upon the animals and to perform a harm benefit analysis of the experiment: Animal experiments may only be authorised if the expected scientific benefit of the procedure is considered to outweigh the expected harm to the animals, because it is only then that they are considered to be ethically acceptable. When evaluating applications, the licensing authorities have to seek advice from ethical review committees, the so-called *"Advisory Committees in accordance with §15 of the German Animal Welfare Act"*.

Due to its de-centralised Federal political structure, in Germany the authorities for licensing animal experiments are located in the regional governments. Each authority has an Advisory Committee assigned to it, which, as a rule, consists of six members, four scientists and two lay members appointed by animal welfare organisations. During their meetings, the committees discuss the indispensability and ethical acceptability of the proposed experiment and convey their opinions to the authorities. In the United Kingdom, the licensing authority is the Home Office. However, in addition, all establishments in which animals are used, bred and/or supplied under the UK Animals (Scientific Procedures) Act 1986, must have a local ethical review process (or committee). This acts as an adjunct to the Home Office providing a *local* framework to ensure that all use of animals is carefully considered and justified; that proper account is taken of all possibilities for implementing the 3Rs; and that high standards of

accommodation and care are achieved. These are also constituted of scientists, veterinary and animal care staff. Some include an independent lay member, but this is not mandatory.

In the presentation, an overview of existing ethical review systems as a part of the authorisation procedure is given, and their strengths, challenges and weaknesses are discussed. From the point of view of animal welfare, the importance of the authorisation procedure and the ethical review process as an integral part of this cannot be overestimated, but the current system does have deficiencies. For instance, there are no well-defined classification systems to determine the severity of the procedures or the significance of the expected scientific benefit. As a result, the harms to animals are frequently underestimated, whereas the benefits are overestimated. Lack of information on replacement, reduction and refinement and lack of knowledge on how to conduct ethical review also stand in the way of the full application of the 3Rs principle in accordance with Directive 86/609/EC.

Report of an animal welfare officer

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In this lecture a short introduction will be presented on the European and Dutch regulatory background and their relation to the position of animal welfare officer (awo) in the Netherlands (NL). At the moment approximately 35 awo's are appointed at the different institutes in NL. According to the Dutch Experiments on Animals Act each licensed institute (e.g. Utrecht University) or industry should charge a competent person (awo) with formal duties to supervise and monitor the welfare of laboratory animals in breeding, in stock or during the conduct of an animal experiment. An overview is given of how the awo is embedded in the University of Utrecht (UU) (and University Medical Centre Utrecht (UMCU)) and how the mandatory duties are implemented. The awo promotes the 3 R's in his tasks: 1. in the education of scientists (course on laboratory animal science), 2. in conducting site visits, 3. in advising the Animal Ethics Committee, the scientists, the license holders (the Rector of the UU and the Dean of the UMCU), the animal tenders, and 4. public relations. How the different tasks are performed will be explained. Since there is an increasing interest into animal welfare topics by the general public, political parties and animal rights movements, there is a growing need for more public (open) information about animal experiments. Public accountability is becoming more and more important for institutes (industries, universities), animal ethics committees, and also for the animal welfare officers in the NL. Current developments in the approach to the duties of awo's will be discussed (professionalization, accreditation).

Introduction to ECVAM and its activities

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ECVAM was created by a Communication from the Commission to the Council and the Parliament in October 1991 (SEC(91)1794) in response to a requirement in Directive 86/609/EEC on the protection of animals used for experimental and other scientific purposes, namely that the Commission and the Member States should actively support the development, validation and acceptance of methods which could reduce, refine or replace the use of laboratory animals.

The main tasks of ECVAM are the funding and management of validation studies and the support to other Commission Services on issues related to alternatives to animal testing. For this purpose, ECVAM has established a broad network of international experts in order to identify the most promising methods and to develop testing strategies, which could reduce, refine or replace animal testing for regulatory purposes, e.g. safety assessment of chemicals (REACH), cosmetics (7th Amendment to the Cosmetics Directive), agrochemicals, pharmaceuticals, biologicals and other products.

The presentation will provide an overview on the principles of validation of alternative methods to animal tests and will highlight activities, which are not specifically presented at this workshop, e.g. carcinogenicity & genotoxicity, reproductive toxicity, ecotoxicology, kinetics and the database on alternative methods.

Recommended articles (to be downloaded from http://ecvam.jrc.it):

- Balls et al. (1995) Practical Aspects of the Validation of Toxicity Test Procedures. ECVAM Workshop Report 5. ATLA 23, 129-147.

- Curren et al. (1995) The Role of Prevalidation in the Development, Validation and Acceptance of Alternative Methods. ECVAM Prevalidation Task Force Report 1. ATLA 23, 211-217.

- Hartung et al. (2004) A Modular Approach to the ECVAM Principles on Test Validity. ATLA 32, 467-72.

- Balls et al (2006) The Principles of Weight of Evidence Validation of Test Methods and Testing Strategies. The Report and Recommendations of ECVAM Workshop 58. ATLA 34, 603-620

ECVAM's Key Area Systemic Toxicity – Summary of ongoing activities

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The overall aim of the key area on systemic toxicity is to validate *in vitro* tests relevant for target organ and target system-specific toxicities to be incorporated into optimal test batteries and strategies for the estimation of human systemic toxicity.

In the area of acute oral toxicity the ICCVAM-ECVAM validation study concluded that 3T3 and NHK Neutral Red Uptake test methods can be used in a weight-of-evidence approach to determine the starting dose for acute oral *in vivo* toxicity studies. The Integrated Project, A-Cute-Tox, aims to improve the correlation between basal *in vitro* cytotoxicity data and rodent LD50 values by introducing correctors such as information on ADME and specific organ toxicity, measured using *in vitro* methods. The ultimate goal is to develop a robust *in vitro* testing strategy for the prediction of human acute oral systemic toxicity of chemicals.

In the area of haematotoxicology, a validation study on CFU-GM assay with rat progenitor cells is ongoing, aiming to increase the accuracy of determining the maximum permissible exposure limit for xenobiotics.

In the area of immunotoxicology a multi-laboratory study has been carried out to evaluate the most promising endpoints for immune-suppression, the results have been published in a peer-reviewed journal and a validation study is ongoing.

In the area of repeated dose toxicity ECVAM is involved in two FP6 projects: Predictomics that aims to develop short-term *in vitro* assays for long-term kidney and liver toxicity, and Pulmo-net that aims to develop advanced *in vitro* methods and technologies for lung toxicity testing.

The final goal in the field of systemic toxicity is to provide cheaper, more ethical and more scientifically based testing strategies.

ECVAM's Key Area Topical toxicity – Summary of ongoing activities

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The European Cosmetics Directive and REACH have accelerated the need for alternative tests. Especially the animal testing ban of cosmetic ingredients from 2009 strongly impacts on the timely delivery of relevant methods.

ECVAM's key area "topical toxicity" focuses on skin and eye irritation/corrosion, phototoxicity and percutaneous absorption. Regulatory accepted alternative tests are available for skin corrosion (OECD TG430, 431), phototoxicity (OECD TG 432) and percutaneous absorption (OECD TG 428).

In the area of skin irritation, ECVAM recently finalised a study that demonstrates the scientific validity of a human reconstituted skin model to fully replace the animal test (see ESAC statements on the ECVAM website http://ecvam.jrc.it).

In contrast, eye irritation testing will probably require an integrated testing strategy combining different *in vitro* assays that altogether might replace the animal test. To this end, ECVAM has contributed to the recent validation of organotypic models for detecting ocular corrosives and severe irritants. In parallel, four promising cytotoxicity- and cell-function- based assays are under evaluation by ECVAM. Finally, ECVAM is planning a prospective validation study on two corneal models using human cells.

Moreover, the key area contributed to the implementation of the REACH legislation by leading the expert group on skin/eye irritation producing technical guidance for industry on toxicity testing under REACH.

Recommended reference (available on the ECVAM website: http://ecvam.jrc.it):

- Eskes C., Zuang V. Eds. (2005) Alternative (non-animal) Methods for Cosmetics Testing: Current Status and Future Prospects. A report prepared in the context of the 7th Amendment of the Cosmetics Directive for establishing the timetable for phasing out animal testing. ATLA 33, Suppl. 1, pp. 227

In vitro human pulmonary barrier models for testing pulmonary toxicants

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The airway epithelium represents a key barrier for the transportation of molecules as well as a potential site for toxicity. The bronchial and alveolar barrier has been shown to play a central role in pulmonary disease. The main aim of the study was to optimize the culture conditions to obtain an in vitro barrier model suitable to study lung toxicity. The first objective is to establish an in vitro functional bronchial barrier; Calu-3, a human bronchial cell line, retains several characteristics of the airway epithelium, such as the capability to form tight junction (TJ) and to produce mucous under air-liquid conditions (AL). Specific parameters were selected to monitor the barrier function: the trans-epithelial electrical resistance (TEER), the permeability of FITC-Inulin and fluorescent staining for TJ (ZO-1, Occludin). The bronchial function was evaluated by checking mucous production by Alcian Blue staining in fixed cells. The second objective is to establish a suitable in vitro alveolar barrier model by co-culturing human alveolar type II (NCI H441) cell line and human pulmonary microvascular endothelial (HPMEC-ST1.6R) cell line to evaluate the toxicity of pulmonary toxicants. Co-culture of NCI H441 and HPMEC-ST1.6R cell lines were grown on opposite sites of microporous polycarbonate filters and the formation of functional alveolar-capillary barrier was investigated by measuring TEER, and by immunostaining of the TJ and the adherens junction proteins, Occludin, ZO-1 and E-cadherin. The specific endpoints were examined in both the mono-culture of the NCI-H441 cell line and the co-culture with the HPMEC-ST1.6R cell line. The investigation of production of surfactant proteins, SP-A, SP-B, SP-C, and SP-D by the NCI-H441 monolayer and by the co-culture model is ongoing. The human in vitro bronchial and alveolar barriers might provide suitable in vitro models to evaluate toxicity of pulmonary toxicants.

ECVAM's activities in the area of *in vitro* neurotoxicity

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A large number of chemicals may exert adverse effects on the central and/or peripheral nervous system. The commonly recommended strategy for neurotoxicity testing is that of a tiered approach aimed at identifying and characterizing the neurotoxicity of a compound. Current OECD and EC guidelines for the evaluation of neurotoxic effects of chemicals for hazard and risk assessment are based solely on *in vivo* studies. To address the problems related to the increasing cost and time required for *in vivo* toxicity testing, the increasing number of chemicals being developed, as well as the current EU legislation where the application of *in vitro* testing strategies is required (REACH, 7th Amendment to the Cosmetics Directive), attention is currently being devoted to the use of *in vitro* alternatives in neurotoxicology.

This presentation will give an overview of ECVAM's activities in the area of neurotoxicology. The available *in vitro* models that range in complexity from simple cell lines (1) to complex reaggregating brain cell cultures (2) will be discussed. The sensitive and cell specific endpoints that enable to assess impaired function of neurons or glial cells and to discriminate between neurotoxicity and general cytotoxicity effects will be reviewed. Especially, the application of new emerging technologies such as measurements of electrical activity using multielectrode array (MEA) (2) and metabolite profiling (metabolomics) and their performance and suitability for neurotoxicity assessment will be discussed.

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ECVAM's Key Area Sensitisation – Summary of ongoing activities

*Silvia Casati – presented by Chantra Eskes**

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In addition to animal welfare considerations, the 7th Amendment to the Cosmetics Directive and REACH require that alternative methods to animal testing for skin sensitization hazard and safety assessments should be urgently developed and validated.

ECVAM's efforts in the field are primarily directed towards this goal. These comprise, at research level, a strong involvement in Sens-it-iv, a EU sponsored integrated project which aims to develop over a period of five years, strategies to replace animal experimentation with *in vitro* assays able to identify skin and respiratory sensitisers.

As well as this, a number of workshops and expert meetings have been and will be organised to address specific topics of interest with a view to help to identity promising methods and to develop recommendations on future research activities.

Emphasis is also given to the refinement and reduction of existing animal tests. Criteria to be used for the assessment of the validation status of modified versions of the standard local lymph node assay (LLNA) encompassing non-radioactive endpoints are being defined. The cut-down LLNA, which halves the number of animals used with respect to the standard test, underwent an ECVAM peer-review process (ESAC statement on ECVAM website: http://ecvam.jrc.it.

The presentation will cover recent and ongoing activities.

The use of alternatives to animal tests in higher education – the UK experience

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In the UK the use of animals for educational purposes has fallen year-on-year from its peak in 1989 (~12,000 or 0.37% of the total used for research) to a low in 2005 (~1600 or 0.06%). This reduction is encouraging, particularly when set against a steep increase in the number of students in the biological sciences, but it is still a significant number, probably a gross underestimate, and probably unnecessary. While a number of bio/medical/health and veterinary courses use animals it is pharmacology courses, and to a lesser extent physiology and biochemistry, which are the main users.

Over this same period there has been a significant trend towards making use of IT in teaching and learning (e-learning) and there are now a wide range of 'proven' non-animal models, including a number of high quality computer-based alternatives, available to teachers. Computer-based learning programs, which simulate such experiments, offer students a virtual laboratory experience and there is now significant evidence that this approach is able to meet many of the learning objectives of laboratory classes though generic and specific laboratory and surgical skills cannot be adequately taught in this way.

Many universities have already adopted computer-based alternatives and are using them in a variety of ways. As a consequence, a typical pharmacology degree course today is likely to contain far fewer laboratory practical classes which use animals than a typical pre-1990 degree course. To further reduce animal use in education it is important to convince and persuade the faculty, who are the key curriculum change-agents. They need to be made more aware of the possibilities that alternatives afford, and they need to be convinced of their educational and cost effectiveness. Teachers also frequently express the desire to be able to modify the computer programs to meet local educational needs and this is something that developers of alternatives need to take into consideration.
This presentation will demonstrate some of the key features of a number of computer-based alternatives to using animals in undergraduate pharmacology teaching developed by the author. It will present evidence of their educational effectiveness, and describe methods to raise awareness of their existence and support their integration into mainstream teaching. It will also outline an ongoing project which is exploring new ways to develop these programs and which provides both for local editing and avoidance of technological redundancy.

Introducing alternatives to using animals in teaching in Balkan Universities

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Although reliable information about animal use in teaching in universities across Eastern Europe is not available, anecdotally it is much higher than in universities in Western Europe as teaching is more traditional and resources to enable adoption of technology and e-learning are scarce. However, over the last few years, teachers in some universities have demonstrated a willingness to embrace new teaching methods.

A project, funded by The Lord Dowding Fund (UK), was initiated in 2006 to explore, with 22 teachers of physiology and pharmacology in Balkan universities, how a reduction in animal use in teaching might be achieved. Funding enabled a range of 17 computer-based alternatives supplied by Sheffield BioScience Programs (www.sheffbp.co.uk) to be freely distributed and a workshop designed to introduce the idea of using alternatives and how these have been used successfully in the UK, was held in Belgrade in September 2006. All participants committed to try to implement at least one of the alternatives into their teaching in the next academic year. They also completed questionnaires to enable collection of reliable information about: the use of animals in education; awareness of e-learning initiatives; current use of alternatives and possible reasons why alternatives were not currently being used.

It is clear from the initial surveys that teachers in Balkan universities use significant numbers of animals in teaching physiology and pharmacology. A minority was aware of e-learning initiatives and none were actively using course management tools such as virtual/managed learning environments. Only 2 of the 22 teachers surveyed were aware of websites which provided information about alternatives to using animals in teaching (e.g. NORINA, AVAR, InterNICHE, EURCA). A large majority felt that computer-based learning offered distinct advantages over traditional teaching methods, that programs could achieve many of the learning objectives of practical classes and that their use would lead to improvements in teaching methods. Interestingly the opinions of their colleagues had the greatest influence on whether they were likely to adopt alternatives.

A follow-up survey is currently in progress and this presentation will report the early results from this. It will also present two case studies of Balkan universities (Belgrade, Serbia and Skopje, Macedonia) where significant reduction in animal use in teaching has been achieved.

ECVAM's guidance on Good Cell Culture Practice

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The maintenance of high standards is fundamental to all good scientific practice, and is essential for maximising the reproducibility, reliability, credibility, acceptance and proper application of any results produced.

Following a suggestion made at the 3rd World Congress on Alternatives and Animal Use in the Life Sciences (1999), ECVAM convened a Task Force with a broad range of expertise in cell and tissue culture, which produced a detailed Guidance on Good Cell Culture Practice (GCCP) for practical use in the laboratory.

The aim of the guidance document is to promote the maintenance of these standards and to reduce the uncertainty in the development and application of animal and human cell culture procedures and products, by encouraging harmonization, rationalization and standardization of laboratory practices, quality control systems, safety procedures, recording and reporting, education, training and compliance with laws, regulations and ethical principles.

This presentation will discuss the Guidance on Good Cell Culture Practice (GCCP) (Coecke et al, 2005) that has been published in ATLA and is being made freely available.

Recommended reference (available on the ECVAM website: http://ecvam.jrc.it):

- Coecke et al. (2005) ECVAM Good Cell Culture Practice Task Force Report, ATLA 33, 261-287

Posters

THE USE OF FIBROBLAST CELL CULTURES AS A PHARMACOLOGICAL TOOL FOR THE INVESTIGATION OF WOUND HEALING ACTIVITY.

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Conventional techniques for the investigation of wound healing activity are mainly based on making in vivo incision and excision wounds in experimental animals. These type of experiments may constitute not only technical difficulties (infection risks, standardization problems, etc.), but also ethical problems. This surgical wounds are, in general, painful situations in animals, even in the case that all experimental procedure have been carried out in sterile and germ-free conditions. Without sterile and germ-free condition, the situation is more troublesome for animals, since the healing period may be prolonged with infection and the pain perceived from animals may be increased. Mainly due to these reasons, alternative methods are necessary for the reduction of animals and the refinement of experimental procedures for the investigation of wound healing activity. Although the pathophysiological role of fibroblast cells in wound healing has been long known, there are only a few cell culture studies using them as a tool for investigation of wound healing. In a previous study, we have investigated the effect of TECA (titrated extract of Centella asiatica, Madecassol) and dexpanthenol (Bepanthene) in mouse T15 fibroblasts using morphometric analysis techniques (1). With this publication, main research outputs were to establish an in vitro experimental model for rapid screening of new drug candidates without using animals and for the investigation of possible mechanism of action in wound healing process. Using similar morphometric analysis techniques in chicken embryonic fibroblasts, we have investigated three secoiridoid compounds isolated from Gentiana lutea ssp. symphyandra, gentiopicroside, sweroside and swertiamarine (2), and St.John's Wort (Hypericum perforatum) extracts (3). These two publications were actually the applications of our experimental model. Although we have no experiments with synthethic compounds as yet, the model can be applied easily for them. In our laboratories, we still have studies for the investigation of some plant extracts, which are known for their wound healing activities. This communication gives detailed information for our studies for wound healing activities using (a) Mouse T15 fibroblasts (b) Human NIH3T3 fibroblasts and (c) Chicken embryonic fibroblasts.

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IN VITRO MODEL FOR RETINAL TOXICITY AND CYTOTOXICITY ASSESSMENT OF NANOTECHNOLOGY BASED LIPOSOMAL DELIVERY SYSTEMS ON CULTURED RETINAL PIGMENT EPITHELIUM CELLS

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The retinal pigment epithelium (RPE) which forms the outer blood-retinal barrier is a target for either intraocularly or systemically administered compounds and the RPE cells in culture provides an in vitro model for studying various effects of drugs. The aim of this study was to evaluate the morphological and electrophysiological properties of RPE cells in culture and to use cultured RPE cells as an in vitro test system for cytotoxicity assessment of nanotechnology based polymer coated liposomal drugs such as Cyclosporine A (CsA) which were prepared for intravitreal applications.
The RPE (D407) cells were cultured in DMEM supplemented with % 5 FBS, glutamine and antibiotics and maintained at 37 ºC in a humidified environment containing 5 % CO2 and the morphology were examined by phase contrast microscopy. In addition, the morphology and ultra structure of RPE cells grown on extracellular matrix-coated or uncoated membranes were examined by electron microscopy whereas electrophysiological properties were evaluated by transepithelial electrical resistance (TEER) measurements. Polymer-coated liposomes between 100 - 200 nm sizes were prepared by using a well established liposome extrusion technique with the aid of different phospholipids, cholesterol, lipopolymer (PEG-DSPE) together with CsA and drug content was analyzed by immunoassay. The RPE cells grown to confluency were treated with different polymer-coated liposomal CsA formulations for 24 - 96 h and free CsA. The cytotoxic effect was determined by WST-1 proliferation assay based on the cleavage of tetrazolium salt by mitochondrial dehydrogenases in viable cells and the cell viability was expressed as the percentage of cell viability of treated cells relative to that of untreated control cells at defined time points.
The RPE cells were proliferated in monolayer with polygonal morphology as observed by phase contrast microscopy. The TEM results confirmed that RPE cells grown on extracellular matrix coated or uncoated membranes in culture were both cuboidal, had microvilli and junctional complexes, although the TEER values were found to be 10.5± 0.766 ohm.cm2 and 9.77 ±0.221 ohm.cm2 respectively. The WST-1 assay results showed that polymer-coated liposomes prepared from phosphatidylcholins with long chain fatty acids dramatically reduced the cytotoxic potential of CsA presenting slowly declined dose-response curves and shift in CsA concentrations applied. However, polymer-coated liposomes prepared from phosphatidylcholins with medium chain fatty acids enhanced the cytotoxicity of CsA at all concentrations tested. These results indicated that nanotechnology based polymer-coated liposomal systems had the potential to alter the cytotoxicity of CsA on cultured RPE cells, but the type of phospholipids or other lipids added to the formulation had important role for increasing or decreasing the cytotoxicity of CsA due to the solubility behaviour of CsA within the liposomal membranes. The RPE cells in culture provide an alternative system for evaluation of retinal toxicity and specific cell based assays on cultured RPE cells with nanotechnology-based delivery systems are in progress at our own cell culture laboratory.

This study was supported by grant from TUBİTAK (Project Nr: TUBİTAK-SBAG 2119) and cell culture studies were conducted at TUBİTAK DNA and Cell Bank-Hacettepe University.

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AN IN VITRO EVALUATION OF VARIOUS ATORVASTATIN SAMPLES FOR THEIR INTESTINAL PERMEABILITIES BY USING PAMPA METHOD

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Assays that predict passive absorption of orally administered drugs have come into prominence especially for early drug discovery and development studies. Due to high cost of drug discovery research, HTS (High-Throughput screening) assays are needed for increasing demand to permeability charecteristics. PAMPA (Paralel Artificial Membrane Permeation Assay) is one of the non-cell based in vitro HTS assays used to predict only passive and transcellular permeability. Various atorvastatin samples, a 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase inhibitor and an antilipidemic drug, purchased from different manufacturers were used for this study. Artificial membrane was created by adding phosphatidylcholine containing dodecane to 96-well filter plates for PAMPA assay and atorvastatin samples were added onto the membrane at donor plate. PAMPA double-sink system was set and acceptor and donor plates were incubated together for 16 hours. Concentrations at acceptor and donor plates were deternimed by UV/vis measurements and Pam, log Pe, Fa, binding percentage of atorvastatin samples to membrane and percentage of transport values were calculated by using permeability equations. The sample which had the best permeability charecteristics was selected for further studies.

EDUCATED SCIENTISTS WILL CEASE THE PAIN OF EXPERIMENTAL ANIMALS

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It is of critical importance to improve the standards of scientific studies where laboratory animal welfare is a must! Marmara University School of Medicine Experimental Research and Animal Laboratory, which was founded in 1988, supplies rats (Wistar and Sprague Dawley strains), mice (Swiss albino) and in vivo experimental facilities to the researchers coming from various departments, faculties and research hospitals. However, good laboratory conditions do not guarantee the humane methods for implementing reduction, refinement and replacement alternatives (3Rs). Ethical justification of laboratory animal use by our institutional committee that was founded in 1996 has served to achieve a greater acceptance of the concept of humane experimental techniques. We also felt ourselves obliged to give education about “The Basic Laboratory Animal and Ethical Principles” and regular atelier studies are being organized since 1999. The objectives of learning in these atelier studies are to get familiar with the laboratory animals, to learn basic physiological properties and husbandry conditions and to get skills in the handling of animals in an ethical perspective. No single animal was sacrificed in these workshops, while invasive techniques, such as tracheostomy, artery cannulation and euthanasia, were presented by video demonstrations recorded during experiments. The members of ethical committee have also prepared a guidebook that contains basic ethical and technical principles.

In a retrospective study, a five-year follow-up of data collected within Marmara University showed that reviewing the protocols and giving feedback to the researchers has an educational value in improving the skills and attitude of persons involved in the design and performance of animal experiments. In conclusion, we suggest that giving education or training and reviewing the protocols will not only decrease the pain of animals but also compunction of the researchers.

AN EXAMPLE OF USING ALTERNATIVE TEST METHODS; IN VITRO CYTOTOXICITY AND GENOTOXICITY OF METALLIC AND POLYMERIC MATERIALS

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Determining *in vitro* cytotoxicity and genotoxicity of implant materials have been very important afterwards CE mark *(European Conformity)* for the manufactured implant materials were obligatory. Tests to ensure the biological compatibility of materials have been specified by regulatory organizations. In this study; four different kinds of metallic and three different kind of polymeric materials were tested according to the test protocols of ISO and OECD. To determine *in vitro* cytotoxicity of materials, cytotoxicity test with biomaterial extract and cell attachment tests were done by following *'ISO 10993-5: 1998 Biological Evaluation of Medical Devices, Tests for in vitro Cytotoxicity' guidlines.* To determine genotoxicity of biomaterials, *in vitro* micronucleus assay with human peripheral blood lymphocytes were done in according with *'ISO 10993-3: 1998 Biological evaluation of Medical Devices, Tests for Genotoxicity, Carcinogenicity, Reproductive Toxicity'* and *OECD Guideline For The Testing of Chemicals Draft Proposal For a New Guideline 487, In vitro Micronucleus Test'.* General design of the tests were done according to *ISO 10993-1:1998 Biological Evaluation of Medical Devices, Evaluation and Testing.* And materials for the tests were prepeared due to '*IS0 10993-12:1998 Sample Preparation and Reference Materials.'*
Standard test protocols for both *in vitro* cytotoxicity and genotoxicity of the international regulatory organizations are routinely applicable in Animal Cell Culture Laboratory at Ege University, Department of Bioengineering for research purposes as well as for the implant material manufacturers.

AN ALTERNATIVE CELL BASED MODEL FOR CHEMICAL INDUCED OXIDATIVE STRESS IN ANIMALS

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Although it is not possible to completely abandon the experimental use of animals in laboratories, increasing public concern as well as ethical issues are forcing researchers to develop alternative approaches giving at least equally reliable models for such studies. Immortalized cell lines, well characterized and proven to be stable, can be promising tools for use in toxicological studies. The relevance of this approach was explored by using wild type Chinese hamster ovary cells (CHO-K1) in the present study. Oxidative stress was induced in cells by menadione and copper plus hydrogen peroxide (Cu^{++}/H_2O_2). The oxidative damage was determined by analysing representative biomarkers for three cellular macromolecules; small chain aldehydes for lipid oxidation (LO), dityrosine for protein oxidation, and 8-OHdG for DNA oxidation. Cells were incubated with the model compounds in the presence or absence of vitamin E and C, and cytotoxicity was evaluated by a nuclear-dye method. Results were compared to two fluorescent probes, H_2DCF-DA and C_{11}-BODIPY$^{581/591}$, which have been used for determining the formation of free radicals in the cells. From ten LPO degradation products, eight were increased significantly following incubation with menadione in cell lysate or incubation media. Menadione-induced oxidative stress was also confirmed by oxidation of fluorescent probes. However, no increased formation of protein oxidation products was observed. Although Cu^{++}/H_2O_2 did not induce oxidation of fluorescent probes, it induced formation of six out of ten LPO degradation products. In conclusion, our present results show that the LPO biomarker set can be used for evaluation of oxidant capacity and the toxic potential of various chemicals. In addition, this *in vitro* cell model proved to be a suitable alternative for such studies conducted in experimental animals.

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