Workshop 5.16
Reproductive toxicology – the EU ReProTect project

Lecture

The future of teratology is *in vitro*

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Birth defects induced by maternal exposure to exogenous agents during pregnancy are preventable, if the agents themselves can be identified and avoided. Billions of dollars and man-hours have been dedicated to animal-based discovery and characterisation methods over decades. We show here, via a comprehensive systematic review and analysis of this data, that these methods constitute questionable science and pose a hazard to humans. Mean positive and negative predictivities barely exceed 50%; discordance among the species used is substantial; reliable extrapolation from animal data to humans is impossible, and virtually all known human teratogens have so far been identified in spite of, rather than because of, animal-based methods. Despite strict validation criteria that animal-based teratology studies would fail to meet, three *in vitro* alternatives have done so. The Embryonic Stem Cell Test (EST) is the best of these. We argue that the poor performance of animal-based teratology alone warrants its cessation; it ought to be replaced by the easier, cheaper and more repeatable EST, and resources made available to improve this and other tests even further.

Lecture

Cross-cutting technologies in the ReProTect project: Objectives and early achievements

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In addition to the development of batteries of *in vitro* assays for the effects on critical components of reproduction, a major aim of the ReProTect EU 6th Framework Integrated Project is to improve the accuracy and predictivity of existing and new assays through the implementation of innovative approaches. An interdisciplinary WorkPackage "Cross-cutting Technologies" is therefore under way and includes the following tasks: development of biosensors, currently targeting receptor interactions; development of QSARs to predict receptor binding as well as transfer of chemicals through blood-testis and placental barriers; implementation of metabolic activation systems to *in vitro* assays for embryotoxicity and endocrine disruption; development of microarray technology as a hazard identification tool for ER-alpha and AR interaction; and, finally, the validation of assays for ER-alpha and AR binding and transactivation, thus representing a major EU component of the OECD validation work. Endocrine disruption also represents a major topic within this WorkPackage, due to its relevance to all areas of the reproductive/developmental toxicology as well as to the overall progression of chemical testing strategies. The outcomes of this WorkPackage will support the other parts of the ReProTect project by providing approaches and experimental models. The achievements of this WorkPackage during its first year include preliminary deliverables in all major areas (e.g., protocols, list of genes for microarrays). Furthermore, two workshops on metabolic activation systems (November 2004, chaired by Prof. Heinz Nau) and sensor technologies (May 2005, chaired by Prof. David Cowell) have been organised.

Lecture

In vitro embryotoxicity of NMP and its three metabolites – NMP interferes with the development of cranial nerves

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Of the three *in vitro* embryotoxicity tests validated to classify the embryotoxic potential of unknown chemicals, only the Whole Embryo Culture (WEC) is able to distinguish between specific dysmorphogenesis of organ primordia and general embryotoxic effects.

The embryotoxic effects of the organic solvent N-methyl-pyrrolidone (NMP), and its metabolites 5-hydroxy-methyl-pyrrolidone (5H-NMP), N-methyl-succinimide (MSI) and 2 hydroxy-methyl-succinimide (2H-MSI) were assessed. Furthermore, underlying mechanisms for occurring dysmorphogenesis in the head region of the cultured embryos were investigated using whole-immuno-staining (WIS) analysis.

9,5-day old embryos were exposed to the test compounds at increasing concentrations. WIS was performed using antibodies specific for cellular-retinoic acid binding protein I (CRABP-I) indicating neural crest cells (NCC) and specific for 2H3-neurofilament indicating Central Nerves (CN). Specific dysmorphogeneses in neurulation and abnormal development of the second visceral arches were induced by NMP and 5H-NMP. WIS revealed disturbed development of CN causing of observed dysmorphogenesis. NMP induced a significantly changed progression of the CN, but did not interfere with NCC. In the order of decreasing embryotoxicity, the ranking of the test substances was as follows: NMP>5H-NMP>2H-MSI>MSI. NMP and 5H-NMP were classified as weakly embryotoxic; MSI and 2H-MSI as non-embryotoxic.

This study suggests that embryotoxicity previously observed after NMP administration to experimental animals is predominantly caused by the parent compound, NMP.

Poster

ECVAM Key Area Reproductive Toxicity: Summary of ongoing activities

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The Key Area Reproductive Toxicity is implemented into an action of the European Centre for the Validation of Alternative Methods (ECVAM), addressing the validation of alternatives for the European chemicals and cosmetics policies. Activities in establishing an ECVAM task force (chair Prof. H. Spielmann) guiding actions in this key area are ongoing. The current activities, targeting embryotoxicity testing based on embryonic stem (ES) cells, neurodevelopmental toxicity and endocrine disruptors are closely linked to the Integrated Project ReProTect, in which ECVAM is responsible for scientific input and the daily management. ReProTect aims for the development and implementation of a tiered testing strategy based on alternative tests for reproductive toxicity. In order to advance this approach, workshops on the test substances selection, metabolic activation, implantation and identification of applicable sensor technologies have been held. Ongoing studies on ES cells embrace further exploitation of the Embryonic Stem Cell Test (EST) and feasibility studies on the transferability to human ES cells. Furthermore, approaches to assess developmental neurotoxicity using different sources of stem cells, namely embryonic, fetal and umbilical cord stem cells are compared. Prevalidation exercises of *in vitro* methods for detection of compounds with (anti)-estrogenic and (anti)-androgenic activity are currently ongoing in collaboration with US EPA and Japan/CERI. In summary, the development, optimisation and integration of *in vitro* models into suitable testing strategies is aimed for, not only to decrease animal test numbers in reproductive toxicity testing, but also to gain more detailed information on the toxicological mechanism in different target tissues.

Lecture

ReProTect – WP2 implantation

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Work Package 2 (WP2) covers the time-window of female reproduction from implantation and maturation of the conceptus till birth, focusing on the process of implantation, including the preparation of the uterine environment, and on placental growth and function.

The main corresponding outcomes from *in vivo* animal studies in this time window, as investigated in 1 and 2 generation studies (described in OECD guidelines), are:

-pre- and post-implantation loss which are measured as low number of pups
-early and late resorptions
-growth retardation

All these negative outcomes found in animal studies are known from human pregnancies as well, occurring "spontaneously" and/or being induced by chemicals such as pharmaceuticals and recreational drugs. These are endpoints where very little information on mechanisms of action is available in the literature, be it in women or in experimental animals.

The human endometrium, the implantation process and placenta formation and function differ significantly from those of rodents, and only some monkey species are fully comparable with humans. To obtain valuable information from *in vitro* test systems, the use of human tissue is thus if not mandatory, at least favourable. This approach is unique among ReProTect sub-projects and can be considered a major input and at the same time a challenge.

WorkPackage II is co-ordinated by professor Lennart Dencker, University of Uppsala – this work package will start after 24 months; new partners will be recruited after the 18 months.

Lecture

EU FP6 Integrated Project "ReProTect" „Development of a novel approach in hazard and risk assessment of reproductive toxicity by a combination and application of *in vitro*, tissue and sensor technologies" Research area III: Prenatal development

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W.P. III.1. Early prenatal development: All test guidelines for regulatory embryotoxicity testing are based on experiments in pregnant animals. Recently, however, several *in vitro* embryotoxicity tests have successfully been validated in an ECVAM validation study. One of them was the Embryonic Stem Cell Test (EST), in which a mouse ES cell line is used, since ES cells are pluripotent and can differentiate in culture into cells of most organs. In the EST the effects of test chemicals on differentiation into beating myocard cell is tested. The EST has the advantage that no embryos or embryonic tissues have to be obtained from pregnant animals. In the ReProtTect project the database of chemicals tested in the EST will be extended and development of ES cell into other major target tissues will be standardised, e.g. nerve cells, cartilage and bone cells. In addition, the potential of human ES cells for embryotoxicity testing will be exploited, since this may allow to avoid problems arising from species specificity.

W.P. III.2. Late prenatal development: Rodent post-implantation Whole Embryo Culture (WEC) is an *ex vivo* test focusing on organ formation during embryogenesis. In the ECVAM validation study the WEC provided reproducible results in four laboratories. However, since metabolic activation of xenobiotics is important for proper determination of toxic effects of chemicals in culture systems, the project will focus on introducing a metabolising system as an adjunct to WEC. Therefore, an existing *in vitro* metabolising system will be introduced as a pre-incubation to the established WEC.