In Vitro Tests for Detecting Chemicals Affecting the Embryo Implantation Process

The Report and Recommendations of ECVAM Workshop 62 — A Strategic Workshop of the EU ReProTect Project

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Preface

This is the report of the 62nd of a series of workshops organised by the European Centre for the Validation of Alternative Methods (ECVAM). The main objective of ECVAM, as defined in 1993 by its Scientific Advisory Committee, is to promote the scientific and regulatory acceptance of alternative methods which are of importance to the biosciences, and which reduce, refine or replace the use of laboratory animals. One of the first priorities set by ECVAM was the implementation of procedures that would enable it to become well informed about the state of the art of non-animal test development and validation, and of opportunities for the possible incorporation of alternative methods into regulatory procedures. It was decided that this would be best achieved through a programme of ECVAM workshops, each addressing a specific topic, and at which selected groups of independent international experts would review the current status of various types of in vitro tests and their potential uses, and make recommendations about the best ways forward (1). The workshop on in vitro tests for detecting chemicals affecting the implantation process and placental toxicity was held in Ispra, Italy, on 2–3 October 2006, under the co-chairmanship of Susanne Bremer and Lennart Dencker. The workshop was built on the reports and recommendations of ECVAM Workshop 53 (2) and on the Research Area of Implantation — ReProTect Workshops held in Copenhagen in January 2005 and in Uppsala in May 2005 (information not published). It was one of a series of strategic workshops within ReProTect, an integrated project aimed at furthering the development of in vitro methods in the area of reproductive toxicology, which consists of a consortium of 32 European partners and is financially supported by the European Commission with Euro 9.1 million. The ReProTect Research Area of “implantation” is aiming to develop tests and testing batteries that are able to detect the effects of chemicals on the various target cells/tissues and mechanisms involved in the preparation of the uterus for the implantation of the embryo, as well as on placentation. The workshop, which was attended by 15 invited participants from six EU Member States,
reviewed a number of possible subendpoints relevant for the process of placental implantation toxicity, and discussed and evaluated alternative methods that could mimic these subendpoints in vitro, according to their test development status and their relevance. The modular approach was introduced and used as a template for the progression of each test system (3). In addition, the workshop participants proposed chemical classes and reference chemicals that should be selected for the use in the development of ReProTect tests.

**Introduction**

After fertilisation, embryonic implantation is a required and critical step in the reproduction of all mammals. The greatest risk to successful pregnancy occurs during the interval from conception to the attachment of the embryo to the uterine epithelium. The essential processes for successful embryonic implantation (including proliferation, differentiation of the endometrium and embryo–maternal signalling) were extensively described more than three decades ago (4). However, the exact molecular mechanism of implantation, the interactions between embryo and endometrial epithelium and uterine stroma, are largely unknown in human beings.

To establish pregnancy, the preparation of the uterus is of vital importance, since it permits embryonic development to continue via physical contact with the maternal uterine tissue through the process of implantation, so that the embryo is provided with oxygen and nutrients, and metabolic waste products are removed (5).

It is known that chemicals can disturb several levels of the implantation process, namely, preparation of the endometrium, synchronisation of blastocyst development and endometrial receptivity, and attachment of the conceptus to the uterus (e.g. the retardation of early postimplantation blastocyst development induced by retinoic acid). In addition, disturbances of hormone balance via xenobiotics could lead to the termination of the pregnancy. Relevant in vivo studies, explicitly one-generation and two-generation reproduction toxicity studies, assess only the number of pups, resorption and growth retardation, but provide no information about the underlying mechanisms of any adverse effects. Within the integrated project, ReProTect, three work packages were defined to analyse toxic effects on the endometrium, the placenta and on the process of implantation. Promising in vitro models are currently being evaluated and combined, in order to achieve a testing strategy that defines the most sensitive target tissues/organs and biological mechanisms involved in impaired implantation (6).

In conclusion, it was the objective of this work-shop, through the willingness of its participants, to identify target tissues/organs/cells and biological mechanisms that are sensitive to chemical insult, prioritise sensitive targets, define toxicological endpoints that can be covered by in vitro testing and identify suitable in vitro models covering other recognised sensitive parameters that will be developed and integrated into testing batteries. It was also important to identify issues which cannot be mimicked by alternative methods.

**Inter-species and inter-individual variation**

**Inter-species variation**

The study of human developmental and reproductive toxicity poses resource, logistical and ethical problems for the use of laboratory animals, due to the high number of animals required for regulatory safety assessments. All the parties involved, both in testing for reproductive toxicity and in the regulatory authorities, are aware of problems in assessing the meaning of results such as teratogenicity in animal tests. An important consideration is whether any observed malformation in animal embryo studies is relevant for risk assessment in relation to human embryos.

An interesting and sad example of the difficulties inherent in data interpretation is the thalidomide disaster (7), which resulted in more than 7000 malformed children. The reasons for this were, firstly, there was no regulation before this drug was introduced, requiring that candidate drugs should be tested for developmental toxicity, so, in that sense, its deleterious effects could not have been prevented; and secondly, rats and mice have been considered insufficient to reproduce the malformations occurring in human babies, so they cannot be considered to be relevant species for predicting such abnormalities. Rabbits, and especially New Zealand White rabbits, respond to thalidomide with various malformations in the limbs, which are partly similar to those seen in human babies. Both in human babies and in rabbits, an array of other malformations were produced as well, and this is the main reason why the use of rabbits has become mandatory for reproductive toxicity testing.

The problem of interspecies variation needs also to be considered when toxic effects on the implantation are discussed. These variations can be exemplified by some key features: a) differences in type of placenta; b) the existence of an inverted yolk sac placenta; c) variation in placental transporter expression; d) divergence of implantation development; and e) difference in endocrine modulation:

a) There are three types of placenta in mammals (8, 9): haemochorial (rodents, humans, and nonhu-
c) The toxicant concentration reaching the embryo is a critical factor in developmental toxicity. Among the mechanisms regulating the disposition of toxicants from the maternal circulation to the embryo, drug efflux transporters play a key role, and are possibly responsible for interspecies variability. An example is mentioned below, under the section on Mechanisms of drug transfer across the placenta (8, 12, 13).

d) Bonnet classified the course of implantation into three categories based on blastocyst–uterine cell–cell interactions, namely: central, eccentric and interstitial (14). Central implantation occurs in mammals such as rabbits, ferrets, and some marsupials; in these animals, the blastocyst grows and expands extensively before implantation. In contrast, the blastocysts of mice, rats, and hamsters are small and show modest expansion. In these species, an implantation chamber is formed by the invagination of the uterine epithelium, which is a characteristic of eccentric implantation. In guinea-pigs, chimpanzees, and humans, the implantation process is of the interstitial type, i.e. blastocysts are embedded within the subepithelial stroma. In addition, Schlafke and Enders (15), on the basis of ultrastructural studies, classified implantation into intrusive, displacement, and fusion types. The first type occurs in humans and guinea-pigs, where the trophoblasts penetrate through the luminal epithelium, reaching the basal lamina and extending through it. The displacement type occurs in rodents; the luminal epithelium is freed from the underlying basal lamina, facilitating the spread of trophoblasts through the epithelium. The fusion type of implantation occurs in the rabbit, where trophoblasts establish a connection with the luminal epithelium by forming symplasma (9, 16).

e) With regard to the endocrine control necessary for implantation, the uterus differentiates to a receptive state that lasts for a limited period, during which it supports blastocyst growth, attachment, and the subsequent events of implantation (17, 18). The major factors that promote uterine receptivity are the ovarian steroids, progesterone and oestrogen. Both hormones are crucial for implantation in mice and rats, but ovarian estrogen is not essential for implantation in pigs, guinea-pigs, rabbits and hamsters (17, 19). Furthermore, in rabbit and pig embryos, the capacity to produce oestrogen has been demonstrated, but whether embryonic oestrogen plays a role in implantation in these species is still debatable. On the contrary, mouse embryos lack the aromatase activity necessary for oestrogen synthesis (20). Whether oestrogen production by the ovary or the embryo plays a role in human implantation is as yet unknown (21).

It appears that humans and animal (rodent or non-rodent) models have some differences in the process of implantation, in addition to the well-known variabilities with regard to the uptake and metabolism of drugs and chemicals (22, 23). These data suggest that there is a high uncertainty with regard to the validity of a risk assessment based on reliance only on animal studies. Therefore, new in vitro models based on human tissues (cell and tissue culture, uterus perfusion, etc.) can provide mechanistic information to address the problem of inter-species variation, and such information can facilitate reliable predictions which are relevant for human hazard identification.

Human inter-individual variation

Genetic variability in human populations poses questions about how and to what degree polymorphism in genes encoding receptors, drug metabolism enzymes and transporters could influence toxic responses to pharmaceuticals, chemicals or environmental pollutants. This characteristic of inter-individual variation needs to be taken into account when developing and validating in vitro models which rely on human tissue/explants or human primary cells, and it generates problems for predicting adverse effects in a highly variable human population exposed to many drugs and other xenobiotics (24). However, there is an increased interest and effort from researchers in developing new alternative methods by using human primates), epitheliochorial (horses, cows, sheep, and pigs) and endotheliochorial (most carnivores). Within these categories, the development of the structure of the placental villi also requires a separate classification (10), into folded (pig), lamellar (carnivores), trabecular (some primates), labyrinthine (rodents, lagomorphs, insectivores) and villous types (humans).

b) Rodents have an inverted yolk sac placenta, which is responsible for the histiotrophic nutrition of the embryo during the first few days of embryonic development. Interference with the function of this placenta, due to the accumulation of a chemical, can cause embryonic death or embryonic toxicity/malformations not occurring in species which lack this placental structure. This can result in the false classification of a chemical as a developmental toxicant (11). This reasoning is, to some extent, also valid for the rabbit. Conversely, there may be examples where the yolk sac placenta may protect the embryo by hindering the access of a chemical to it.
material, since these models will improve the relevance of the results produced (25). To enhance these benefits, scientists are developing new alternative approaches for evaluating the toxicological implications of human genetic variability in response to xenobiotics, such as physiologically-based pharmacokinetics modelling (PBPK) which incorporates information on polymorphism into the analysis of toxicokinetic variability (26).

Taking into consideration all the characteristics of inter-species and inter-individual variation, a more-scientific and more-mechanistic, rather than a routine, approach to toxicity testing should be emphasised, whereby each piece of data can be evaluated in terms of its relevance to the corresponding human toxicological effect. The numerous ad hoc assumptions that provide a rationale for these generic approaches to reproductive risk assessment can only be evaluated critically when mechanistic data are available, which are also relevant to the low-dose exposure, and when inter-species extrapolation problems are considered. There is a clear need to employ the available animal and human data bases, and to develop new alternative methods for in vitro assays and in vivo tests by applying refinement and reduction principles, and explicitly incorporating them into the risk estimation process.

The Identification of Potential Toxicological Target Organs/Tissues/Cells and Biological Mechanisms

A successful embryo implantation requires a perfect synchronisation between the development of the blastocyst and the receptive endometrium, which will allow the attachment and the invasion of the embryo into the differentiating stroma. Changes in the architecture of the endometrium, as well as in the expression of molecules and specific structures (pinopodes) on the epithelial cell surface, have been observed, leading to a receptive status of the uterus for implantation (27). These findings might be used to establish new toxicity test models for the identification and detection of chemicals which affect the implantation process.

A prominent example of an embryonic marker is the early pregnancy factor, human chorionic gonadotrophin (hCG), which is a recognised indicator of successful implantation. Many other implantation markers are, as yet, insufficiently characterised. Therefore, it has been claimed that research on markers of implantation should be intensified (28). In this context, several factors representing endometrial proliferation and receptivity, blastocyst function and embryo–maternal signalling, might be addressed as possible toxicological endpoints for implantation failure. Examples of putative markers could be the various adhesion molecules that are expressed in endometrial and blastocyst cells. The most predictive endpoints should be selected from among the following parameters, in order to develop reliable toxicity test models: the steroid receptors, progesterone receptor alpha (PR alpha) and oestrogen receptor alpha (ER alpha), leukaemia inhibitory factor (LIF; 20, 29–36), integrins (e.g. αvβ3), calcitonin, insulin growth factor-binding protein-1 (IGFBP-1), cyclo- oxygenase-2 (COX-2), 15-hydroxyprostaglandin dehy- drogenase (PGDH), corticotropin-releasing hormone receptor (CRH-receptor 1), vascular endothelial growth factor-A (VEGF-A), VEGF receptor 2 and kinase insert domain receptor (KDR; 37–42).

Other molecules have been discovered as important mediators of the implantation process, which could facilitate the development of new testing methods, such as the vasoactive agents, histamine and prostaglandins (PGs). These molecules play an essential role in implantation and in pro-inflammatory reactions, increasing vascular permeability at the site of blastocyst implantation and decidualisation (43, 44, 45–52). It would also be very valuable to consider the expression of various growth factors (e.g. IGFBP-1) and their receptors, since there is evidence of their presence in the uterus in a temporal and cell-specific manner during the peri-implantation period (5, 53–58), as well as a number of various cytokines (e.g. LIF, interleukin-1β [IL-1β]) and their receptors, which are present in the uterus and in the embryo during early pregnancy (59, 60). There are also newly-discovered receptors, such as the (endo)-cannabinoïd receptors, which have been found in the uterus during early pregnancy (61, 62), which may have a role in implantation.

Many of the above-mentioned putative markers have only been investigated in animal models, and their significance for embryo implantation in humans has yet to be elucidated. However, they should be studied as a basis for the development of new alternative methods that would have scientific and ethical advantages, being based on mechanistic information, especially if the models employ human cell lines or primary cells. This would avoid, or at least reduce or refine, the use of animals, resulting in reliable toxicity test models that would be rapid and cost-effective.

The Assessment of In Vitro Methods Relevant to the Implantation Process

Several in vitro systems with the potential to be used as predictive tests for the detection of chemicals affecting the placental implantation process, have been identified for integration into the ReProTect project, with the aim of optimising their respective protocols and evaluating their predictive capacities for providing specific toxicological information relevant to embryo implantation (Table 1). Each assay has been evaluated according to ECVAM’s evaluation criteria (3), in order to demon-
<table>
<thead>
<tr>
<th>Name of the test</th>
<th>Explanation of mechanism basis</th>
<th>Definition of endpoint predicted</th>
<th>Standard operational procedure</th>
<th>Test is precisely</th>
<th>Test performance criteria are described</th>
<th>Limitations are described</th>
<th>Training set of chemicals are listed</th>
<th>Definition of prediction model is given</th>
<th>Method is used in other laboratories</th>
<th>Domain of applicability</th>
<th>Assessment of reproducibility of experimental data in same laboratory</th>
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</thead>
<tbody>
<tr>
<td>Human endometrium explants</td>
<td>Y</td>
<td>PR, ER, LIF, calcitonin, integrin (e.g. αvβ3), IGFBP-1, COX-2, PGDH, CRH-R1, IL-1, VEGF, KDR to be considered</td>
<td>N</td>
<td>N</td>
<td>Mifepristone, tamoxifen, COX-2-inhibitor, nioxine, DES</td>
<td>Y (endometrium availability, accuracy of endometrial dating, inter-individual variation, heterogenous tissue, drug metabolism variation)</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
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<tr>
<td>Human chorionic villi explants</td>
<td>Y</td>
<td>Viability, hCG, hPL, proliferation (Ki-67, BrdU); caspase-3 expression, MMPs</td>
<td>Y</td>
<td>Y</td>
<td>pNP (p-nonylphenol)</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
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<tr>
<td>BeWo cells</td>
<td>Y</td>
<td>Viability, hCG, hPL, proliferation (Ki-67, BrdU); caspase-3 expression, MMPs</td>
<td>Y</td>
<td>Y</td>
<td>pNP (p-nonylphenol)</td>
<td>N</td>
<td>N</td>
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<td>JEG 3 cells</td>
<td>Y</td>
<td>Viability, hCG, hPL, proliferation (Ki-67, BrdU); caspase-3 expression, MMPs</td>
<td>Y</td>
<td>Y</td>
<td>pNP (p-nonylphenol)</td>
<td>N</td>
<td>N</td>
<td>N</td>
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<tr>
<td>HTR-8/SVneo cells</td>
<td>Y</td>
<td>Viability, hCG, hPL, proliferation (Ki-67, BrdU); caspase-3 expression, MMPs</td>
<td>Y</td>
<td>Y</td>
<td>pNP (p-nonylphenol)</td>
<td>N</td>
<td>N</td>
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<td>N</td>
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<td>N</td>
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<tr>
<td>Placental perfusion</td>
<td>Y</td>
<td>Transfer, expression of stress proteins, DNA adducts, enzyme activity (EROD, CYP1A1, etc)</td>
<td>Y</td>
<td>Y</td>
<td>For transfer assessment: Antipyrine (+), radioactive heparin/immunoglobulins (-) For toxicity: to be determined by further research</td>
<td>Y (perfusion time, tissue viability, technical skills)</td>
<td>Cadmium, nioxine, carbamazepine/ oxazepam, benzo(a)pyrene, glyphosate</td>
<td>Y</td>
<td>Y</td>
<td>Y</td>
<td>Antipyrine: data available</td>
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<tr>
<td>Human endometrial-endothelial cells</td>
<td>Y</td>
<td>Proliferation, BrdU, PCNA, viability, vital staining, LH release</td>
<td>N</td>
<td>N</td>
<td>o,p'-DDT, PCB 77, PCB 126, DBP, BPA, TCDD, sex hormones</td>
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<td>N</td>
<td>N</td>
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<td>N</td>
<td>N</td>
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<tr>
<td>Human placental pericyte/endothelial cell co-culture</td>
<td>Y</td>
<td>Viability, differentiation</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
<td>N</td>
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Y = currently available, N = not yet available. PR = progesterone receptor; ER = estrogen receptor; LIF = leukaemia inhibitory factor; IGFBP-1 = IGF-binding protein-1; COX-2 = cyclooxygenase-2; PGDH = 15-hydroxyprostaglandin dehydrogenase; CRH-R1 = corticotropin-releasing hormone receptor 1; IL-1 = interleukin 1; VEGF = vascular endothelial growth factor; KDR = vascular endothelial growth factor receptor 2 (VEGFR2), also known as KDR; hCG = human chorionic gonadotropin; hPL = human placental lactogen; Ki-67 = human Ki-67 protein (strictly associated with cell proliferation); BrdU = bromodeoxyuridine; MMPs = matrix metalloproteinases; EROD = ethoxyresorufin-O-deethylase; CYP = cytochrome P450; PCNA = proliferating cell nuclear antigen; DES = diethylstilbestrol; o,p'-DDT = o,p'-dichlorodiphenyl trichloroethane; PCB = polychlorinated biphenyl; DBP = di-n-butyl phthalate; BPA = bisphenol A; TCDD = 2,3,7,8-tetrachlorodibenzo-p-dioxin.

1Positive (+) and negative (−) controls; 2method restricted to specific chemicals; 3done by participants, not by an independent statistician.
strate which additional optimisation steps need to be performed before the tests can be considered suitable for formal validation studies.

Mechanisms of drug transfer across the placenta

Detailed understanding of the mechanisms employed in the transfer of drugs across the placenta is crucial for the optimisation of pharmacotherapy in pregnant women. In general, any chemical substance administered to the mother is able to permeate, to some degree, across the placenta (63, 64). The placenta had long been viewed as a passive barrier through which chemical compounds pass, based on their physical-chemical properties and the concentration gradient. It has been agreed that non-ionised and lipid molecules with molecular weights of up to 600 Daltons cross the placenta via passive diffusion. However, over the last two decades, several drug efflux transporters and biotransformation enzymes have been shown to form an “active component” of the placental barrier, which has brought new and important insights into the field of transplacental pharmacokinetics. These proteins are directly involved in the protective role of the placenta, by limiting the disposition of drugs and xenobiotics to the fetus.

Over the last decade, increasing effort has been devoted to the investigation of ATP-Binding Cassette (ABC) drug efflux proteins in the placenta, including P-glycoprotein (P–gp, multi-drug resistance gene [MDR1]) encoded by the ATP-binding cassette, sub-family B member 1 [ABCB1]), multidrug resistance-associated proteins (MRPs encoded by ATP-binding cassette, sub-family C [ABCC1–6 and ABCC10–12]) and breast cancer resistance protein (BCRP, encoded by ATP-binding cassette, sub-family G [ABCG]). Some of these transporters were originally found to be associated with multidrug resistance in cancer cells, but are currently also recognised for their substantial roles in the modulation of drug absorption, distribution and metabolism (65–67).

P–gp, MDR1 was the first-discovered, and so far is the best-characterised, of the drug efflux transporters, whose role in the regulation of drug disposition to the fetus has been extensively studied (68). The expression of placental P–gp has been confirmed at both the gene and protein levels by a range of experimental approaches. In the study of Cordon-Cardo et al. (69), intensive immunoreactivity for P–gp was revealed in the trophoblast layer, whereas the endothelial cells of placental fetal capillaries were proved to be P–gp negative. Strong MDR1 expression, comparable with that in the intestine and liver, was further confirmed in the human placenta at the mRNA level (70). In rats, P–gp expression over the course of pregnancy showed a tendency to increase toward term (11), while in humans, a different pattern was observed (12). Discrepancies among species could be explained by the species differences in the mechanisms involved in the regulation of P–gp expression. The functional activity of P–gp was described by using cell models (71–73) and animal models (74–76), as well as in vitro perfused human cotyledon preparations (77–79).

High expression of BCRP in human placenta was originally reported by Allikmets et al. (80), who named the transporter “placenta-specific ATP-binding transporter”. In subsequent studies, BCRP was found to be localised at the trophoblast surface layer of placental chorionic villi (81, 82). Experiments performed in transgenic mice revealed the importance of BCRP for fetal protection (83, 84). Most recently, the functional activity of BCRP has been confirmed in microvillus membrane vesicles prepared from the human placenta (85) and in the BeWo human cell line (86). It was therefore suggested that placental BCRP may, like P–gp, contribute to the protective function of the placenta, while transporting cytotoxic drugs and possibly toxic xenobiotics out of the placenta. In addition, BCRP expression in the human term placenta was found to be more than one order of magnitude higher than that of P–gp-encoding MDR1 (86). Direct evidence for the function of BCRP as an active component of the materno–fetal barrier has been recently provided in the dually-perfused rat placenta by Staud et al. (87). In this study, BCRP was also shown to actively remove its substrate from fetal circulation to the mother against a concentration gradient.

The family of MRP transporters currently comprises nine members (MRP1–9) that are involved in transport of a broad range of substances, including organic anion drugs, glutathione, glucuronate and sulphate conjugates, nucleotides and their analogues (88–91). The expression of most of the MRP members has recently been detected in human and rat placental tissue (92, 93). At the protein level, MRP1 was found on the basal membrane of the human placental trophoblast (94, 95), which is in contrast to the localisation of MRP2 at the apical syncytiotrophoblast membrane (90). Together with MRP3, MRP1 was also detected in the endothelia of fetal blood vessels (96). Most recently, the gestation age-dependent expression of MRP5 was observed in the human placenta (97). Although it seems attractive to presume that MRPs are involved in the protection of the fetus, as are P–gp and BCRP, their role in the placenta remains to be clarified.

Drug metabolism enzymes in the placenta and endometrium

The human placenta is known to express a spectrum of metabolising enzymes involved in phase I,
as well as in phase II, biotransformation reactions (98). With reference to drug metabolism, of the known human cytochrome P450 (CYP) enzymes, functional activity in the placenta has been confirmed for the CYP1A and CYP2E1 isoforms. Several phase II enzymes (e.g. sulphotransferases, glutathione-S-transferases, uridine diphosphate-glucuronosyltransferases, and N-acetyltransferase) have also been found to be active in placental tissue. To date, however, these enzymes have not been proven to play an important role in detoxification (98), with the exception of placental 11β-hydroxysteroid dehydrogenase, which is capable of deactivating glucocorticoids in both the placenta and in the fetal circulation (99).

The human endometrium is also reported to express various phase I and II enzymes, which may be of importance in relation to the effects of drugs and chemicals in the human endometrial explant model (100, 101). Hukkanen and coworkers have reported that neither CYP1A1 nor CYP1A2 mRNAs are expressed in endometrial samples (102). The CYP1 family is, however, modulated by arylhydrocarbon receptor (AhR) ligands, including persistent environmental pollutants and tobacco smoke (103). The AhR receptor is expressed in the endometrium (104), and CYP1A1 and CYP1B1 protein levels are strongly up-regulated by AhR ligands in explant cultures of the human endometrium (105). Immunohistochemistry has demonstrated that CYP1B1 protein is detectable in the stroma and glandular epithelium (100), and that the expression is higher during the proliferative phase than during the secretory phase (106). The CYP2 family is also expressed in human endometrial samples (102), and the endometrial level of CYP2E1 mRNA correlates well with levels of serum oestradiol (107). The CYP3A subfamily has also been detected in endometrial samples, and the mRNA expression correlates with the oestrous phase (102, 108). Immunohistochemistry and in situ hybridisation have indicated the expression of CYP2C8 and CYP3A proteins in the stroma and glandular epithelium (100, 108). Also, phase II enzymes, such as UDP-glucuronosyltransferases (UGTs) and sulphotransferases, are expressed in the human endometrium (109, 110). This expression may vary with the phase of the menstrual cycle, as well as being affected by the use of oral contraceptives (111, 112).

Taken together, the data indicate that there is a low but distinct expression of phase I and II drug metabolism enzymes in the human placenta and in the endometrium. The cellular expression of endometrial drug metabolism enzymes should be examined in more detail, and the ability of the human endometrium to bioactivate various model toxicants remains to be examined, before endometrial explants can be proposed as a human in vitro model for the testing of drugs and chemicals.

**Uterine function**

**Human endometrial cell culture**

The human endometrium has a complex structure, which includes luminal and glandular epithelial cells, stromal cells, endothelial cells and immune cells. The regulation of endometrial function depends on the endocrine action of steroid hormones such as oestrogen and progesterone, and paracrine communication carried out via the actions of various growth factors and cytokines among the different cell compartments. Therefore, studies on the endometrium demand a variety of culture methods for assessment of the function and regulation of endometrial receptivity and the implantation process.

The two main cell types in the human endometrium are stromal cells and epithelial cells. These two cell types can be separated and cultured individually or in co-culture systems. Epithelial cells can be cultured as primary cultures or as cell lines. The availability of endometrial tissue and the relatively slow growth of primary endometrial cells during culture has led to the use of cell lines such as the Ishikawa cell line. These cells have receptors for oestrogen and progesterone, and are therefore assumed to act as do primary cell cultures. The advantage of using a cell line is that it is easily accessible and can be cultured for several passages. The major disadvantage is the loss of normal differentiation (113).

For implantation studies, the mouse blastocyst has been used to study the attachment of an embryo to the endometrial surface (114).

Stromal cells undergo decidualisation from the mid-secretory phase and during pregnancy. Decidualisation can be induced in vitro by using oestrogen, progesterone and dibutyryl cAMP (cyclic adenosine monophosphate; 115). In addition, it is possible to culture stromal cells as primary cell cultures, or as immortalised cultures by using simian virus transfection or telomerase expression (116, 117). Data on the use of endometrial cells in toxicology testing are limited. However, it has been shown that the mycotoxins, alpha-zearalenol (alpha-ZOL) and beta-zearalenol (beta-ZOL), influence the phosphorylation state of mitogen-activated protein kinases (MAP kinases), protein kinase B (Akt), and also have effects on eukaryotic translation initiation factor 4E (eIF4E) and eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1; 118).

For the preparation of a co-culture system, the cells are separated by using enzymatic digestion with trypsin and collagenase (119). Thereafter, the stromal cells are cultured in a layer of collagen, placed on a culture insert. The epithelial cells are cultured on a thin layer of Matrigel (120). The advantage of using a co-culture system is that epithelial and stromal cells are well defined,
which permits the study of the various cell interactions (121–123). By using this system, it is also possible to study the initial attachment phase of the implantation process in vitro (120).

**Human endometrial explant culture**

Explant cultures (124–126) are a closer approach to in vitro conditions than are primary or permanent cell cultures, since the integrity of the tissue and communications among its cells are maintained and there is less dedifferentiation of the glandular and stromal components. Furthermore, in explants, some cell types, such as endothelial cells and cells of monocyte–macrophage origin, which are important for cellular communication, are still present.

The explant culture model has been applied in several pharmacological or toxicological studies to investigate effects of exogenous substances (e.g. mifepristone, α-hydroxytamoxifen, nonoxynol-9 and 2,3,7,8-tetrachlorodibenzo-p-dioxin [TCDD]) on morphological and functional parameters of the human endometrium (127–130). Other tissue culture applications include investigations of the factors involved in endometrial physiology and pathophysiology (131–134).

Endometrial tissues can be obtained by aspiration curettage (e.g. Pipelle) from premenopausal women with benign gynaecological disorders, who have had no hormonal treatment for at least three months. Explants (approximately 1–2 mm cubed) are usually cultured for 24 hours, and test chemicals are administered during this incubation period. The time-point in the menstrual cycle at which the biopsy had been taken can be determined by different methods, e.g. by histological dating (135, 136) or by recording the day of luteinising hormone (LH) surge (125). The implantation window corresponds to day 5 to day 10 after the LH surge (38). After the implantation window, non-pregnant endometrium displays an increased sensitivity, due to its preparation for menstruation by beginning apoptotic or necrotic processes (125).

The use of human endometrial explant cultures presents some experimental problems and limitations, due to the use of small amounts of hormone-dependent tissue available from cycling women. Thus, there arise questions on the precise timing in the cycle at which a sample was obtained, the precise localisation in the uterine cavity from which it was taken, and the proportions of epithelium, stroma, blood vessels and immune cells in individual specimens (135, 137–139). Morphological methods are, however, useful to reveal cell-specific changes. Furthermore, endometrial factors having a role in the later stages of the implantation process cannot be evaluated in an endometrial model alone, since these processes are also dependent upon signals from the blastocyst (140).

**Endometrial-endothelial primary cells**

Human uterine tissues contain several different cell types that express steroid receptors, which make them potential targets for drugs, chemicals and endocrine disruptors. One important constituent of the endometrium is the vascular tree, which is renewed during each menstrual cycle. Normal vascular morphology and function are of the utmost importance for bleeding control and fertility. The endometrial blood vessels also have a key role during implantation and placentation in early pregnancy.

The endometrial blood supply is provided by the arcuate arteries, from which radial branches penetrate inward and divide into straight and spiral arterioles. The straight arterioles nourish the basal layer of the endometrium. The spiral arterioles nourish the functional layer with its glands and stroma, and are sex steroid responsive. After menstruation, the functional layer is regenerated, and new spiral arterioles are formed from the straight arterioles in the basal part of the endometrium. The formation of new capillary blood vessels from pre-existing microvessels is known as angiogenesis (141). The female reproductive tract is the main site of angiogenesis in healthy adult humans (142, 143).

The vascular endothelial cell has a central role in all angiogenic processes. The human endometrial-endothelial cell (HEEC) is unique among endothelial cells, in the sense that it expresses ER alpha and possibly also PR (143–146). Endometrial angiogenic activity is under the overall control of ovarian steroids via cyclical changes in oestrogen and progesterone concentrations during the menstrual cycle (147). During and immediately after menstruation, angiogenesis is promoted by the expression of potent angiogenic stimulators, such as vascular endothelial growth factor A and fibroblast growth factor-2 (148).

Angiogenesis can be achieved by different mechanisms, as reviewed by Gargett and Rogers (149). After activation of the endothelial cells by some form of angiogenic stimulus, new blood vessels form by sprouting, incorporation of circulating endothelial progenitor cells into growing vessels, intussusceptive microvascular growth, or vessel elongation. In the endometrium, the main angiogenic mechanism is believed to be vessel elongation, accompanied by intussusception and the incorporation of circulating endothelial progenitor cells (150).

Endothelial cells cover the luminal surface of all blood vessels, and control the vascular morphology and functions through processes such as angiogenesis, vascular remodelling and functional changes. 3,3′,4,4′-tetrachlorobiphenyl (PCB 77) and 3,3′,4,4′,5-pentachlorobiphenyl (PCB 126) compromise the normal functions of porcine vascular endothelial cells by increased expression of the CYP1A1 gene, oxidative stress, and DNA-binding activity of nuclear factor kappa B (NF-κB; 151).
Furthermore, PCB 126 and 2,3,7,8-TCDD affect the cardiovascular system in rats (152).

The expression of ER beta and PR might also render HEECs susceptible to endocrine disrupting chemicals (EDCs). The recently discovered non-classical transmembrane oestrogen receptor (153) offers another possible pathway for endocrine disruption, if it is present in the HEEC.

Since endothelial cells are known to be important for endometrial function, and are probably involved in some common endometrial pathologies, such as menstrual bleeding disturbances (153) and endometriosis (154, 155), in vitro studies can facilitate the identification of the various different effects and mechanisms whereby sex hormones and endocrine disrupting chemicals affect HEECs.

The in vitro culture of human endometrial-endothelial cells is relatively easy to perform. Endometrium can be obtained from hysterectomy specimens from women in the proliferative or secretory phases of the menstrual cycle. After isolation, HEECs cultivated in vitro express the oestrogen and progesterone receptors, at least up to passage five (144). To demonstrate an endothelial cell-specific response, Olovsson and co-workers are currently investigating several markers, such as hypoxia-inducible factor 1 alpha (HIF-1α), VEGF-R2, VEGF-R3, clusters of differentiation molecules (CD31, CD34), von Willebrand factor (vWD), stress proteins, and transporter proteins. The use of these characteristics may well make this model ideal for studies on endothelial cell responses to chemicals, drugs or environmental contaminants, as well as assisting in the development of promising new alternative testing methods.

Placentation

Microvascular fragment models

In many ways, the placenta has been neglected in relation to its role as a target organ in reprotoxicity testing. The lack of knowledge of mechanisms of action in the placenta is remarkable and a hindrance to understanding of the role of placental disturbances in embryo toxicity. The placenta is also neglected when it comes to using placental tissues and cells in toxicological testing, where it may have great potential.

For most of the first half of human pregnancy, the placenta is growing, a process dominated by trophoblast invasion of the uterine tissues and by vasculogenesis. The vascularisation of the human placenta is achieved by a local de novo formation of small vessels derived from pluripotent mesenchymal precursor cells inside the placental villous core, rather than by angiogenesis via sprouting from already-existing vessels (156).

Two main cell types are involved in vasculogenesis, namely, endothelial cells and pericytes. Pericytes are in close contact with the endothelial layer of the vasculature, playing a role in the exchange of nutrients and oxygen across the vessel. They can contract, and control capillary blood flow by regulating vascular diameter. Manipulating pericyte function may cause physiological disturbances similar to those which occur in some human diseases (157).

During angiogenesis in the embryo, sprouting endothelial cells form the new blood vessel channel, then, through the production of PDGF-B, attract PDGFRβ-expressing pericytes that line the newly-formed vessel. This is one paracrine signalling circuit between the pericytes and endothelial cells, but it also believed that pericytes can transfer angiogenic signals to the endothelial cells. This process is essential for the development of a functionally-correct vessel. It has been shown that mouse embryos lacking either PDGF-B or PDGFRβ have vasodilation, which causes oedema, severe haemorrhages and embryonic lethality, suggestive of a central role for pericytes in vascular integrity (158). More recent research on endothelium/pericyte interactions has been published (159), including signalling not only by the platelet-derived growth factor (PDGF)-B system, but also via transforming growth factor beta (TGF-β), angiopoietin, sphingosine-1-phosphate, and other factors.

Pericytes have been shown in vitro and in vivo to differentiate into various cells types, so it has been suggested that they function as multipotent mesenchymal stem cells. There are some criteria that should be fulfilled to define a stem cell: they should be undifferentiated cells, capable of proliferation to maintain the stem cell population and to produce a large number of differentiated, functional progeny, and able to regenerate the tissue after injury (160). By isolating microvascular fragments (MVFs) from human placentas, it is possible to derive primary pericyte cultures. Differentiation from a stem/progenitor cell through a number of transit cell states, and finally differentiation to a fibroblast can be followed (J. Karén and C. Sundberg, in preparation). Thus, they are human stem cells, they are of fetal origin, they are easy to obtain in great amounts and to grow in vitro, they can maintain their capacity to metabolise xenobiotics in a physiological way, and there are relatively straightforward ways of quantitatively monitoring their early stages of differentiation.

In vitro human trophoblast cell systems

Various in vitro models (161, 162) have been developed for studying the dynamics of the trophoblast during placentation. These include the use of established cell lines (BeWo, JEG-3, HTR-8), and primary cultures such as isolated trophoblast cells and
chorionic villous explants. Unlike primary cultures, the established cell lines have the advantage that they can be propagated indefinitely in vitro. They exhibit many similarities, but also some differences, with respect to trophoblast-specific gene expression (163). For these reasons, it would be advisable to use established human cell lines to set up the most appropriate culture conditions, which could be later be confirmed with primary cultures.

Among the primary culture models, preference should be given to the chorionic villous explants. Unlike isolated trophoblast cells, they have the advantage of maintaining the paracrine inter-relations between the different cell components (164, 165). Other advantages of this model are: a) high cell viability (162); b) no enzymatic disruption of the villous structure (164); and c) short time of development (165). Taking all these characteristics into consideration, this in vitro system could be a suitable model for the study of the effects of chemicals that interfere with the processes involved in implantation (166).

Human placental perfusion method

Human placental perfusion (i.e. retaining the viability of an organ or part of an organ with artificial circulation) has long been used for studies on placental physiology and on drug kinetics in and through the placenta (167, 168). However, studies on environmental compounds by using this method are just beginning (169, 170). The same principles and difficulties apply in all placental perfusion studies, and it is advisable to take into account all previous experience in the further development of the method toward an in vitro test system.

The placenta can be, and has been, perfused in many ways. According to the structure and circulation of the placenta, the most physiological system is double-sided perfusion, where both the maternal and fetal circulations are simulated. Perfusion fluid can be pumped through the placenta continuously, via a closed tubing system or in open, so-called once-through, perfusion system. The latter experiments are usually carried out for short periods, and the former can be used for longer, up to 48-hour, perfusions (171). Dual systems are based on a recirculating perfusion, which involves using clear buffer-based perfusion medium in short perfusions (up to 2 hours), and cell culture medium-based perfusion fluid to maintain the tissue for longer periods (172). The perfusion equipment consists of two perfusion pumps for each of the separate circulations (maternal and fetal), double-walled glass chambers for keeping the tissue and perfusion medium warm, and a water pump for the circulation of heated water in the system and tubing network, with perfusion medium reservoirs for flow, sampling and adjustments. Blood gases, pH and the volume of perfusion medium are repeatedly monitored during the perfusion (173, 174).

Another important aspect of perfusion studies is that samples taken can include the tissue, as well as the perfusion fluid (168). Tissue samples from the placenta can be taken only before and after perfusion, but perfusion fluid can also be sampled during perfusion. In this last case, the concentrations of the test compound and the transplacental transfer which takes place, can be easily evaluated. Putative metabolites can also be analysed, to reveal the metabolic capacity of the placenta (175). The toxic responses of the placental tissue, of both the trophoblast and the vascular endothelium, can be studied, as well as the accumulation of the test compound, metabolites and the binding to macromolecules, e.g. DNA-adducts. Finally and very importantly, a comparison of several placentas can also be used assess inter-individual variations in pharmacokinetics.

The Current Assessment of Reproductive Toxicity for Regulatory Purposes

The current regulatory data requirements for assessing hazard to the reproductive system vary considerably, depending on the test compound and estimated exposure to it. The information requirements are most stringent for agents where exposure is deliberate and for those that are designed to be bioactive. These include pharmaceuticals and pesticides/biocides. For industrial chemicals, data requirements increase progressively with increasing production volumes or when other, non-reproductive, studies reveal indications of possible reproductive effects.

The standardised animal tests used for hazard assessment focus mainly on a number of key phases and events of the reproductive cycle. Nevertheless, these landmarks do not provide specific information on the different aspects of the reproductive cycle. They are largely designed as apical tests, where observations such as “litter size” stand for, and are taken to cover, fertilisation, implantation and prenatal development. Nevertheless, the in vivo test is the only test procedure currently accepted by the regulatory authorities.

Due to the considerable progress in science and toxicology, a more evidence-based approach can be expected in the future. This knowledge is certainly necessary for predicting adverse effects to human beings, and will increase consumer and patient protection (176). The relevance of implantation as a target for reproductive/developmental toxicity is a typical example of what is not adequately covered by the currently accepted regulatory tests. OECD Test Guideline (TG) 416 (Two-generation reproduction toxicity study) or OECD TG 421 (Reproduction/developmental toxicity screening test) only
Safety Precautions when Working with Human Tissue

Human tissues such as blood and placenta present infection risks to the personnel involved in handling them. The Human Immunodeficiency Virus (HIV), Hepatitis B (HVB) and Hepatitis C (HVC) viruses and other pathogens are of most concern in relation to occupational injury (177). The World Health Report published data in 2002, which demonstrated that 2.5% of HIV cases and 40% of HVB and HVC cases among health care workers are the result of occupational exposure. Researchers and technicians who collect and process blood and other human tissues are also subject to risk, the magnitude of which is unknown (178). The incidence of laboratory infections has been reported to be decreasing during the past decade (179), partly due to HVB vaccination, but this is not universal (180). Preventive safety precautions must be taken to limit the transmission of infectious diseases from donated tissue to the personnel handling it.

Blood and body fluid exposure is defined as any percutaneous (needle stick injury, cut) or mucocutaneous (splash to the mucosa of the eyes, the mouth, or to intact skin) exposure to blood, a blood-soiled biological fluid, or a fluid known to transmit blood-borne pathogens (181).

Vaccinations

In most European and North American countries, employees with a high level of blood exposure are offered HVB vaccination. It is not possible to immunise against HVC and HIV, which leaves safety equipment as the only precaution available at the present time (182). If an injury occurs, it is important to report and initiate post-exposure prophylaxis against HIV infection, which has to be initiated immediately. Undiagnosed HIV and HVC in patient tissues are possible, and are not always reported in medical records. HIV is not measurable during the first period of infection, and the symptoms of HVC often emerge 20–30 years after viral infection. Hospital personnel have to be instructed not to hand over tissues from cases of HIV, HVB or HVC, to avoid their use in a research project. Since researchers normally do not have access to the medical records, the responsibility has to be with the hospital at which the material is obtained.

Administrative policies

In a critical review of the literature on sharps injuries (182), it is recommended that nurses need to be provided with information regarding the risk factors that are known to be associated with such injuries, and measures that can be employed to prevent infection if injury occurs. Administrative policies prohibiting the practice of recapping must be instituted, as well as a written plan for controlling potential exposures. A policy requiring that all sharps injuries must be reported is needed. Management should ensure that there is adequate staffing, a secure work environment, and an adequate supply of the safest equipment available. These policies should be implemented in the laboratory and among laboratory staff working with blood.

Personnel

In addition to vaccination, it is important that the personnel handling blood and tissue are trained in the equipment they are using. Hollow bore needles and suture needles are responsible for most percutaneous injuries (183). If the needles are contaminated with blood, they represent the highest
infection risk. All procedures must to be known to the staff, who must be trained before handling blood with the risk of infection. Safety instructions regarding the waste disposal of blood-contaminated materials must be known to, and rigorously implemented by, all the personnel. If the laboratory personnel have contact with patients (e.g. during the collection of blood), it is important to apply the same safety precautions toward the patients, as infectious diseases may be transmitted from equipment to them.

Safety equipment

Laboratory coats, gloves and safety goggles should be worn at all times when blood or tissue are being handled. This can prevent contact between the skin and blood, and between the eyes and blood. In the case of wounds or scratches, extra precautions must be taken. The skin should be covered tightly, and double protection should be worn. Needles, scissors or scalpels engineered to be safer than conventional equipment, should be used. Proper deposit-and-disposal boxes for waste materials can also help to reduce the hazards associated with sharp devices used by oneself or by others (184).

Waste and cleaning

Tissue waste or materials contaminated with blood must be discarded separately. Materials contaminated with blood must not include sharp devices, such as glass or suture needles. Sharp devices must be collected and discarded in a deposit box made of hard plastic. Cleaning and the removal of waste from the laboratory must also to be performed by individuals wearing safety equipment. All laboratory utensils must be flushed before ordinary cleaning, and all surfaces must to be cleaned thoroughly with 70% ethanol before and after use (185).

Ethical Issues

The use of human tissues for research purposes and as models in tests requires informed consent from donors, according to the ethical standards laid down in Helsinki declaration (186), as well as the approval of local ethics committees. Ethical approval must be obtained for use of the following models: primary cells, co-culture with primary cells, tissue explants, and organ perfusion.

The principle of autonomy in relation to enrolment, non-maleficence by participation in minimising pain and discomfort, and data protection, is relevant to the Implantation WP (WP2) within the ReProTect project. The study persons will not themselves benefit from donating tissue, so their donations represent altruism for the benefit of society as a whole. Study protocols must be submitted to the ethics committees prior to initiation of the study, in accordance with local regulations, and the procedure for obtaining informed consent should be included, along with other written information.

Ethics approvals for WP2 were obtained for studies on immunoregulatory peptides in materno–fetal immune tolerance, the effects of environmental pollutants on cultured cells from the human uterus and ovary, and experiments with the placental perfusion model. The approvals include permission to use human endometrium from healthy fertile women, obtained by biopsy in the clinic or during the sterilisation operation. The endometrium is shed at the end of each menstrual cycle. This means that the endometrium that is obtained for research would have been shed during the next menstruation, and thereby would have been lost naturally by the donors anyway. The pain is similar to menstrual pain, and little bleeding results from the operation. Another approval permits the use of human embryos, specifying that they should not be suitable for use in the clinic (i.e. could not be transferred).

Another important aspect comprises data protection, where, in accordance with the law on personal data, all research projects which include sensitive personal information must be notified to the appropriate data surveillance authority (e.g. the national data inspectorate).

Finally, the Research Ethics Committee requires that the protocol must include proper information concerning the study persons, the retrieval of informed consent from each study person, respect for the right to withdraw from a study at any time in the study programme without losing treatment equal to that given to other persons, and respect for the right to know or not to know about the study results (186). Any sensitive personal information must be notified to the appropriate data surveillance authority (e.g. the national data inspectorate).

Recommendations for Research and Development

1. The identification of in vitro systems that can simulate placental transfer, especially in early pregnancy, is highly relevant to the study of reproductive toxicity. The further development of the available models by using stably transfected cell lines, such as BeWo human cells (different clones, characterisation of cells regarding transporter expression, etc.), needs to be taken into consideration, as does a comparison of in vitro transfer data with the results from established placental perfusion models.

2. From present knowledge, it is not possible to recognise specific ‘reference chemical compounds’
for the study of implantation toxicity. The identification and classification of such chemicals requires a literature search, with particular attention paid to organs/tissues such as the placenta and the endometrium for the implantation process. The identification of ‘negative reference substances’ is also crucial for a probable further validation exercise. The ReProTect project should organise a detailed literature search to these ends.

3. The immune system has a relevant function in implantation-related physiological processes, and must be taken fully into consideration in evaluating the effects of chemicals on embryo implantation. Since the participation of the immune system is difficult to mimic in in vitro models, the workshop participants reached an agreement that an additional expert meeting and collaboration with other ReProTect project partners is needed.

4. The development of in vitro test models based on human endometrial cells or tissues should be encouraged. These test models should address predictive toxicological endpoints in both the proliferative and secretory phases of the menstrual cycle. This requirement is based on the importance for implantation of a well-differentiated, oestrogen-primed endometrium at the end of the proliferative phase.

5. Diseases such as pre eclampsia and endometriosis, which have a high incidence in the human population, might affect embryo implantation and placentation. Whether chemicals play a role in the aetiology of these diseases is largely unknown. These themes also need extensive investigation, and the ReProTect project should organise a detailed literature search.

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