

Chapter 10: Reproductive Toxicity

Introduction

Reproductive toxicity refers to the adverse effects of a substance on any aspect of the reproductive cycle (Figure 10.1). These include the impairment of reproductive function; the induction of adverse effects in the embryo, such as growth retardation, malformations, and death; and the induction of adverse post-natal effects. Conventional animal tests for reproductive toxicity include the pre-natal developmental toxicity study (OECD Test Guideline [TG] 414 [1]), the one-generation study (OECD TG 415 [2]), the two-generation study (OECD TG 416 [3]), the reproductive/developmental toxicity screening test (OECD TG 421 [4]), and the repeat-dose toxicity study combined with the reproductive/developmental toxicity study (OECD TG 422 [5]). In recent years, there has been a considerable increase in the number of short-term *in vivo* screening tests carried out in the context of reproductive toxicity testing, to detect so-called “endocrine disruptors” (6, 7). This topic is discussed further in Chapter 11.

Scientific Background

Because it is not possible to model the whole of the reproductive system *in vitro*, the parts of the system need to be studied individually. A number of useful and promising *in vitro* systems are now available.

Gametogenesis

Gametes are derived from the primordial germ cells (PGCs), which enter the gonads during pre-natal development. The PGCs arise at some distance from the presumptive gonads, to which they migrate and where they become established. The formation of the germ line is dependent upon the presence of the germ plasm, a cytoplasmic component that makes these cells distinct from the somatic cells. When the PGCs are established in the gonad, they form stem cells that divide by mitosis to produce the supply of male and female gametes for reproduction. In the gonads, the germ cells associate with specific somatic cells that support, nurture and protect them. In the female, these somatic cells are the follicle cells. In the male, the comparable somatic cells are called Sertoli cells. During the proliferative phase, the germ cells are called oogonia in the ovary and spermatogonia in the testis. They act as a stem

cell population that divides by mitosis to produce a life-time supply of gametes for reproduction. The gonial cell divisions may be incomplete, with the result that the daughter cells maintain inter-cellular communication with one another via connecting bridges. Successive incomplete divisions produce very large clones of interconnected cells. This intercellular communication may serve to synchronise the development of the conjoined cells. When the organism reaches maturity, the germ cells acquire the ability to differentiate into functional gametes by meiosis, reducing the chromosome number from $2n$ to $1n$.

Oogenesis

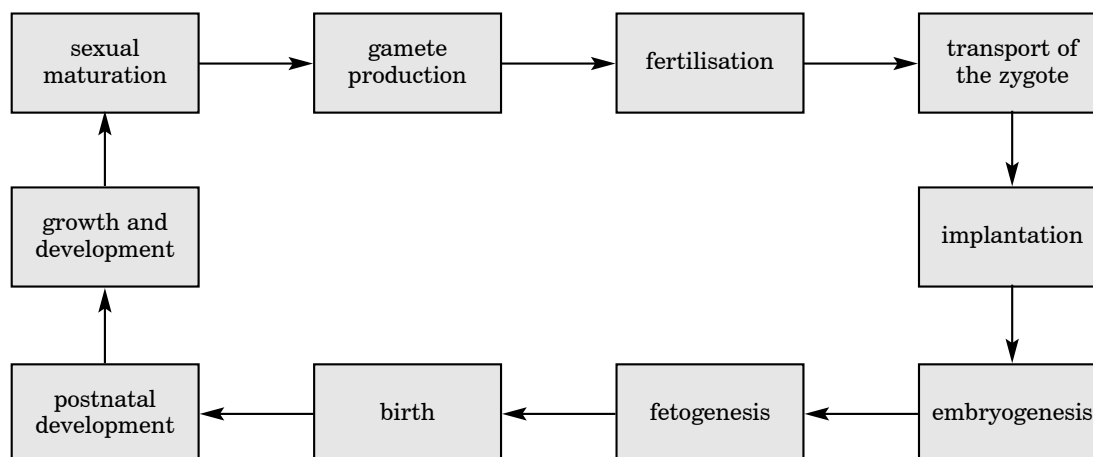
During the first meiotic division, the primary oocyte divides to produce one small polar body and one secondary oocyte. The latter will enter the second meiotic division to produce the second polar body and the haploid ovum, which is the only functional sex cell to result. In most mammalian species, differentiation of the oocyte occurs during a protracted prophase of the first meiotic division. The resumption of meiosis, which occurs after the oocyte is fully grown, is called oocyte maturation.

Theca cells and granulosa cells

The ovaries provide three types of hormone: testosterone, oestrogen and progesterone. The theca cells that surround each follicle make testosterone. Some of the testosterone is released into the bloodstream, but most of it is taken up by the granulosa cells that form the follicle and convert testosterone to oestrogen. As the follicles grow, more and more oestrogen is produced, and the level of oestrogen in the bloodstream rises. After ovulation, the empty follicle turns into the corpus luteum. This produces progesterone, which is important because it prepares the uterus for implantation. Progesterone is only produced in the second half of the cycle, and its detection in the bloodstream indicates that ovulation has occurred.

Spermatogenesis

In the male, meiosis precedes sex cell differentiation. A single spermatogonium enters the first meiotic division as a primary spermatocyte. This division produces two secondary spermatocytes,

Figure 10.1: The mammalian reproductive cycle

The figure excludes behavioural aspects and the fetal/maternal interactions.

each of which divides to form two haploid spermatids. Each spermatid then differentiates (by a process called spermiogenesis) into a spermatozoon through specific structural and functional differentiation, which enables the sperm to fertilise the egg. Consequently, four haploid sperm cells result from each diploid spermatogonium. The utilisation of all four haploid cells in the male is significant, since the testis must produce millions of sperm simultaneously.

Leydig cells and Sertoli cells

Functional compartments within the testis can be defined in terms of their roles in the development of gametes or in steroid hormone production. The Leydig cells in the interstitial tissue of the testis are positioned so as to provide testosterone for the seminiferous tubules, which drive spermatogenesis, and to the peripheral circulation, which supplies testosterone to the rest of the body, to form and maintain male secondary sexual structures and characteristics.

Sertoli cells are non-proliferative, columnar epithelial cells extending from the basal lamina to the lumen of the seminiferous tubule. Adjacent Sertoli cells are united by band-like, occlusive (tight) junctions, which divide the seminiferous tubule into a basal compartment which extends from the basal lamina to the Sertoli cell tight junctions, and a luminal compartment which extends from the tight junctions to the lumen of the seminiferous tubule. Developing sperm cells occupy deep recesses in the lateral surfaces of Sertoli cells.

The blood–testis barrier

The basal (outer) compartment of the seminiferous tubule is accessible to substances within the circulation (blood), whereas the adluminal (inner) compartment is not accessible to substances in the blood.

As gonocytes differentiate into specialised mature sperm cells, they move across the blood–testis barrier.

The placental barrier

The placenta is a materno-fetal vascular organ, which is formed during the implantation of the blastocyst, and is delivered with the fetus at birth. During pregnancy, it provides nutrition, gas exchange, waste removal, and endocrine and immune support for the developing fetus. The main function of the placenta is to permit the exchange of substances, rather than to act as a barrier. Most drugs and infectious agents readily pass across the barrier. The placenta produces several hormones, including chorionic gonadotrophin and the steroid hormones, progesterin and oestrogen.

A general guideline for the administration of drugs during pregnancy is that whatever the mother receives, the fetus will also receive by diffusion across the placental barrier. However, the transport and metabolism of drugs in the placenta are poorly understood processes, because experimental studies with animals are difficult, and studies on humans are impossible. Furthermore, there are significant interspecies differences in placental function.

Current Status of Alternative Methods for Reproductive Toxicity

An overview of alternative methods for reproductive toxicity testing is presented in Table 10.1. More-detailed information is available in ECVAM workshop report 12 (8), and in a review

article by Spielmann (9). The following sections on the developing embryo and the placental barrier address *in vitro* methods for developmental toxicity, whereas the sections on PGCs, germ cells, sperm motility and morphology, and Leydig and Sertoli cells, cover other aspects of fertility.

Table 10.1. An overview of *in vitro* methods for reproductive toxicity

Target cells/ organs	Test System	Endpoint	Applicability	Formal status
PGCs	Primary cells PGC/EGC cell lines	Viability, sister chromatid exchange, mutagenicity	Germ cell mutagens	Further development required
Spermatogonia	Primary cells Cell lines (GC-1 spg)	Specific gene expression	Chemicals affecting fertility	Further development required
Spermatocytes	Primary cells	Differential display	Chemicals affecting fertility	Further development required
Semen	Primary cells	Sperm motility, motion, morphology	Chemicals acting postmeiotically	Ready for prevalidation
Sertoli cells	Cell lines (15P-1, TM 4)	Cell survival/growth	Chemicals destroying the blood–testis barrier and the support of germ cell maturation	Further development required
Leydig cells	Leydig tumour cell lines (MA10; RC2, TM3, MLTC), and genetically engin- eered HEK 293 cells with LH receptors	Cytotoxicity, proliferation, apoptosis, steroidogenesis, Western/Northern blotting, steroid dehydrogenesis	Chemicals interfering with endocrine function (endocrine disruptors)	Readiness for prevalidation needs to be assessed
Fetal oocytes	Primary cells	Viability, specific gene expression	Chemicals affecting fertility	Further development required
Thecal cells	Primary cells	Not developed	Chemicals interfering with endocrine function (endocrine disruptors)	Further development required
Granulosa cells	Primary cells	Not developed	Chemicals destroying the support of germ cell maturation	Further development required
Blood–testis barrier	Sertoli cell lines (TM 4)	Not developed	Chemicals crossing the barrier	To be evaluated by ECVAM
Placenta and placental barrier	Trophoblast cell lines (BT1)	Not developed	Chemicals inducing placental toxicity	To be evaluated by ECVAM
Developing embryo	WEC, MM, EST	Morphological endpoints and cytotoxicity	Direct acting embryotoxic/ teratogenic compounds	Scientifically validated and endorsed by the ESAC

The developing embryo

Birth defects and malformations in humans can be inherited or can result from exposure of the mother and the embryo to drugs, occupational and environmental toxicants, or to other ingested substances, during critical and sensitive periods of development.

In the field of developmental toxicity, a variety of alternatives to animal testing are available. ECVAM has funded the prevalidation and validation of three embryotoxicity tests: the whole embryo culture (WEC) test, the micromass (MM) test, and embryonic stem cell test (EST; 10, 11). For each assay, a prediction model has been developed, to classify the chemicals into three classes of embryotoxicity (non, weak, strong). The scientific validity of the three methods was endorsed by the ECVAM Scientific Advisory Committee in October 2001: the EST and the WEC test were considered to be scientifically valid for distinguishing between non, weak/moderate and strong embryotoxins, whereas the MM test was considered scientifically valid for identifying strongly embryotoxic chemicals (12–14).

In 1998, the US Environmental Protection Agency (EPA) asked ICCVAM to evaluate the frog embryo teratogenesis assay — *Xenopus* (FETAX), a 96-hour, whole-embryo, developmental toxicity test involving the anuran amphibian *Xenopus laevis*. In June 2001, an expert scientific committee concluded that FETAX is not sufficiently validated or optimised for regulatory use (<http://ecvam.niehs.nih.gov/methods/fetaxdoc/>).

The placental barrier

Audus and his colleagues are planning to investigate how drugs of abuse interact with the placenta *in vitro*, by using cell culture models of the placental barrier, representing the early, middle, and terminal stages of pregnancy (15).

Another *in vitro* system, based on a fibre-based bioreactor design, uses a three-dimensional cell culture model that more closely mimics the trophoblast environment *in vivo*. An initial inoculum of trophoblast cells has been maintained in this system for over four weeks. Periodic removal and examination of samples of cells demonstrated that they readily attached to the fibrous matrix and formed three-dimensional structures reminiscent of their *in vivo* architecture (for example, villous-like structures). Examination of the fibrous matrix by electron microscopy demonstrated that the trophoblast cells contained a complement of organelles similar to that found *in vivo*, indicating that the cells had achieved some degree of morphological differentiation. Metabolic studies of glucose consumption and lactate accumulation indicated that the culture reached a steady state within about 2.5

days, and that the production of 17β -oestradiol was maintained indicated further metabolic activity.

Primordial germ cells

The *in vitro* maturation of germ cells in humans is a challenging process to model, because gametogenesis is a lengthy process, encompassing many complex cellular changes. PGCs migrate from the base of the allantois to the genital ridge. They proliferate both during migration and after their arrival, until the fetal gonads start to undergo sexual differentiation. The PGCs then enter into the prophase of the first meiotic division in the ovary to become oocytes (16), whereas those in the testis become mitotically arrested to become prospermatogonia.

The regulation of the growth of mouse PGCs has been studied by culturing them on feeder cell layers. They show a limited period of proliferation *in vitro* before entering growth arrest, which corresponds with their developmental changes *in vivo*. However, in the presence of multiple growth signals, PGCs can restart rapid proliferation and transform into pluripotent embryonic germ cells (EGCs). Studies on ectopic germ cells and on reaggregate cultures have shown that both male and female PGCs undergo cell-autonomous entry into meiosis, and differentiate into oocytes if they are separated from the male gonadal environment (17). Further research is necessary to determine the extent to which some of the existing PGC lines (18) retained the characteristics of PGCs (19).

In addition, two-dimensional dispersed culture systems have been developed, in which the transition from the mitotic PGCs into the leptotene stage of the first meiotic division can be analysed. Entry into meiosis seems to be programmed in PGCs before they reach the genital ridges, unless it is inhibited by putative signals from the testicular somatic cells.

Spermatogonia and oocytes

An ambitious project was funded by the EC in the Fourth Framework Programme. The aim of the study was to establish germ cell culture systems and relevant markers for toxicological endpoints (20). Primary oocytes and spermatogonial cells were cultured *in vitro* and exposed to germ cell toxicants with cytotoxicity, apoptosis and selected gene expression as toxicological endpoints. It was demonstrated that the *in vitro* model is more sensitive than the *in vivo* system. Unfortunately, no standard operating procedure or *INVITTOX* protocol has been developed, so the system cannot be considered to be ready for prevalidation (21–23).

Another project on the production of oocytes (entitled *In vitro production of high-quality mam-*

malian oocytes for biotechnology, assisted reproductions, breeding and toxicology-teratology purposes) is under way. A positive outcome for this study could lead to a source of oocytes that could be used for toxicological studies.

Sperm motility and morphology

Gametogenesis is a complex biological process that is sensitive to environmental insult, for example, from chemicals. Chemical effects on germ cells and their maturation can inhibit fertility, and may have negative effects on the development of the offspring. Mutagens, for example, produce heritable gene mutations, and heritable structural and numerical chromosome aberrations in germ cells. The consequences of germ cell mutation for subsequent generations include the following: genetically determined phenotypic alterations without signs of illness; reduction in fertility; embryonic or perinatal death; congenital malformations with varying degrees of severity; and genetic diseases with varying degrees of health impairment.

Semen analysis permits the detection of the effects of chemicals on post-testicular stages. This kind of analysis is important in two ways: a) changes in sperm or seminal content may be indicative of adverse effects on the male reproductive system, with possible implications for fertility potential; and b) defects in sperm DNA or chromosomes may be associated with subsequent changes in viability during embryonic development, with health risks to the offspring.

Several techniques are available for monitoring sperm motility, motion, morphology, head morphology, chromatin structure and various other aspects of semen composition (24). Some of these analyses can be automated (for example, by videography). A practical system has been well developed and an *INVITTOX* protocol is available (24–26).

Leydig cells and Sertoli cells

There have been some recent claims that there is evidence of a deterioration in mammalian (including human) male reproductive health, leading to suggestions that a number of chemicals found in the environment act as endocrine disruptors. In addition, a wide range of compounds have been reported to be toxic to one of the pivotal cell types involved in spermatogenesis, namely, the Leydig cell (27).

A test system for detecting the adverse effects of chemicals and environmental pollutants on male fertility was developed in the EC's Fourth Framework Programme. Intercellular interactions in the testis have been modelled by using co-cultures of Leydig and Sertoli cells (28). Leydig cell

lines showing constitutive activity in steroidogenesis have been established, and several toxicants have been tested with regard to their effects on steroidogenesis, cell proliferation and cell viability (29, 30). ECVAM is planning to follow up this work by organising a prevalidation study on the use of Leydig cell lines.

Additional research on the blood–testis barrier is required, to establish and characterise the chemical classes that are able to cross the barrier and affect germ cell maturation. An understanding of the mechanisms operating in this barrier could help to decrease the number of possible germ cell mutagens that have to be tested (31–34).

Projects Funded by the European Commission

ECVAM has funded, or is funding, the following studies:

1. The ECVAM prevalidation and validation studies on three alternative tests for embryotoxicity (10–14).
2. An investigation on reporter gene expression in embryonic stem cell-derived embryoid bodies as an endpoint for identifying potential embryotoxic/teratogenic substances.
3. *In vitro* methods for metal-induced infertility and spermotoxicity.

The projects that have been funded by DG Research are summarised in Table 10.2. ECVAM is in contact with the coordinators of some of these projects, to discuss the possibility of follow-up ECVAM prevalidation studies.

Reproductive Toxicity: Summary, Conclusions and Recommendations

Because it is not possible to model the whole of the reproductive cycle *in vitro* with one approach, the parts of the system need to be studied individually and then integrated into testing strategy.

For the assessment of embryotoxic potential, the EST, the WEC and the MM tests can be used immediately. However, the EST is the only one of the three which could have a relatively high throughput, and which would not involve the killing of large numbers of pregnant animals.

It will take time to develop and validate a battery of alternative tests that can cover the various aspects of the reproductive cycle, so animal tests will continue to be required for the foreseeable future, at least for certain aspects of reproductive toxicity testing. Therefore, a short-term priority

Table 10.2: Projects on reproductive toxicity funded by the European Commission (DG Research)

1.	Development and evaluation of Leydig cell lines as <i>in vitro</i> models for toxicological testing	Project Reference: BIO4-CT97-2022 Start date: 01.10.1997	End date: 30.09.2000	Duration: 36 months
2.	Development of 3D <i>in vitro</i> models of human tissues for pharmacotoxicological applications	Project Reference: BIO4-97-2148 Start date: 01.09.1997	End date: 31.08.2000	Duration: 36 months
3.	Identification of critical rat testicular genes altered after fetal androgenic disruption by flutamide: use of DNA microarray approach	Project Reference: QLK4-2000-00684 Start date: 01.01.2001	End date: 31.12.2003	Duration: 36 months
4.	<i>In vitro</i> production of high-quality mammalian oocytes for biotechnology, assisted reproduction, breeding and toxicology-teratology purposes	Project Reference: QLK3-1999-00104 Start date: 01.02.2000	End date: 31.01.2003	Duration: 36 months
5.	Development of <i>in vitro</i> mammalian germ cell culture systems and genetic markers for reproductive pharmacotoxicology	Project Reference: BIO4-CT96-0183 Start date: 1.10.1996	End date: 30.09.1999	Duration: 36 months
6.	Development of a new test of developmental toxicology	Project Reference: Start date: 01.11.1993	End date: 31.10.1996	Duration: 36 months
7.	The use of gene transfer technology in conjunction with primary and clonal culture for the establishment of predictive <i>in vitro</i> screening assays for teratogenic potential: development, validation and transfer to industry	Project Reference: BIO4-CT96-0183 Start date: 01.09.1993	End date: 31.08.1996	Duration: 36 months

should be to review existing animal tests for reproductive toxicity in order to reduce the numbers of animals required and minimise animal suffering.

Short-term prospects (developmental toxicity)

1. The transfer of the EST to interested laboratories.
2. The development of test guidelines based on the EST, WEC and MM tests, and strategies for their use to be submitted for regulatory acceptance.
3. The refinement of the EST with target cell-specific endpoints and human embryonic stem cells.

Medium-term prospects (developmental toxicity)

A catch-up validation study, to assess the validity of the refined EST.

Long-term prospects (developmental toxicity)

The development and evaluation of a testing strategy that covers not only malformations, but also other manifestations of developmental toxicity, such as growth retardation and embryoletality.

Short-term prospects (fertility)

1. The identification of the most predictive toxicological endpoints for use with semen analysis.

2. The prevalidation of a Leydig cell line test.

Medium-term prospects (fertility)

The prevalidation of methods for metal-induced infertility and spermotoxicity.

Long-term prospects (fertility)

1. The development and evaluation of a testing strategy covering all essential aspects of the male and female reproductive cycles.
2. The validation of a Leydig cell line test.
3. The validation of methods for metal-induced infertility and spermotoxicity.

Recommendations for research and development

1. An evaluation of the use of primary cultures of spermatogonia and oocytes.
2. Research on the use of PGCs and/or PGC lines for identifying germ cell mutagens.
3. The further development of granulosa cell and theca cell assays as toxicological screening systems.
4. An evaluation of the use of Sertoli cell lines (including Sertoli cell co-cultures) for toxicological purposes.
5. Further research on the blood–testis barrier, to define its permeability characteristics and the chemical classes that can cross it.

Other recommendations

ECVAM should organise workshops on:

1. embryotoxicity, to define the areas of application of the scientifically validated EST; and
2. the use of hormone-producing cells for predicting the adverse effects of chemicals on fertility.

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