Review of Chemical Toxicity to the Reproductive System, with Particular Reference to Developmental Toxicity

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ABSTRACT

This review was conducted in support of HPA Strategic Goals 2 and 5, 'to protect against the adverse effects of acute and chronic exposure to chemicals, poisons and other environmental hazards' and 'to protect and improve the health of children', respectively.

The possible effects of environmental chemicals on human reproduction and the development of the unborn child is an emotive issue and it is important that the HPA retains a contemporaneous knowledge of scientific developments in this contentious area. It is important when considering this subject to appreciate that the aetiology of congenital abnormalities is a complex, multifactorial event: the majority of congenital abnormalities are not considered to arise from chemical or other environmental exposures. Even with an exposure to a chemical of concern, many other factors may influence the development of an abnormality; such as the timing of the exposure in the pregnancy, the genotype of the unborn child and mother or maternal health and dietary status. These and other factors are discussed.

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Executive Summary

This review was conducted in support of HPA Strategic Goals 2 and 5, 'to protect against the adverse effects of acute and chronic exposure to chemicals, poisons and other environmental hazards' and 'to protect and improve the health of children', respectively.

The possible effects of environmental chemicals on human reproduction and the development of the unborn child is an emotive issue and it is important that the HPA retains a contemporaneous knowledge of scientific developments in this contentious area. It is important when considering this subject to appreciate that the aetiology of congenital abnormalities is a complex, multifactorial event: the majority of congenital abnormalities are not considered to arise from chemical or other environmental exposures. Even with an exposure to a chemical of concern, many other factors may influence the development of an abnormality; such as the timing of the exposure in the pregnancy, the genotype of the unborn child and mother or maternal health and dietary status. These and other factors are discussed.

The available experimental methods for investigating toxicity to all aspects of the reproductive system are outlined. These include a number of regulatory and experimental studies that use animals and form a key component in current chemical safety assessments. However, consideration is also given to the development of new approaches that use *in-vitro* techniques. These may be used as pre-screens in the assessment process to identify priority compounds warranting a more detailed consideration. This approach is also consistent with the aims of reducing, refining and replacing animal use in research.

A number of chemicals are known to produce adverse effects on reproduction or development following exposure during pregnancy and are classified in the EU on the basis of these properties. These are listed. However, it is recognized that there are gaps in our knowledge of the potential toxicity to the reproductive system of many of the chemicals to which the public may be exposed. This knowledge gap will be addressed to some extent by the recently agreed EC Regulation on the Registration, Evaluation and Authorisation of Chemicals (REACH). It is important that the HPA is aware of the outputs from this regulatory process.

Although there are a number of recognized chemical teratogens, their underlying mechanisms are largely unknown. Some generic pathways in human development that are felt to warrant further investigation are listed in the review. It is clear that an understanding of normal embryological and fetal development is important to this subject area.

Epidemiology studies seeking to establish an association between exposure to environmental chemicals and toxicity to the reproductive system have not provided definite evidence for any causal relationship, although there is suggestive evidence in a number of areas that warrant further consideration. Such studies are difficult to interpret because of limitations in the available data on the relevant health statistics and on exposure to chemicals of concern together with the need for careful control for any confounding factors.

There are a number of factors that the population is exposed to which are known to affect the unborn child including excessive alcohol consumption, smoking and many illegal drugs of abuse. Issues surrounding maternal health status include the adequate

intake of nutrients during pregnancy. Folic acid is one of particular interest, as deficiencies have been associated with neural tube defects such as spina bifida. In the absence of further efforts to reduce or control known risk factors in the population, it will remain difficult to identify critical exposures.

Contents

Document Identification 1

Executive Summary 2

Contents 4

"Review of Chemical Toxicity to the Reproductive System, with Particular Reference to Developmental Toxicity" 5

Introduction 5

Section 1: Methods of Assessing Chemicals for Reproductive and Developmental Toxicity 7

In Vivo Study Designs: OECD Guidelines on Assessment 8
In Vitro Study Designs 14
In Vitro Assays for Embryotoxicity 14
Novel Strategies for In Vitro Assessment 18
European Community ReProTect Project; Future Work on the Validation of Alternative Methods; 19
Conclusion on Testing Strategies in Developmental Toxicity 20

Section 2: Chemical Teratogenesis 21

Teratogens and Teratogenesis 23
Mechanisms in Chemical Teratogenesis 27

Section 3: Chemicals Implicated as Reproductive Toxicants; Consideration of the Evidence of Environmental Exposures 33

Classification of Reproductive Toxicants 33
Consideration of Evidence Regarding Environmental Exposure 38
Disinfection by-products 38
Landfill Waste Sites 39
Government Research Relating to Landfill Sites and Health Effects 40

Section 4: Detection of Effects on Fertility after Chemical Exposures 41

Infertility and Sperm Damage Assessment 42
Specific Biomarkers for Toxicity to the Male Reproductive System 43
Selected Biochemical Indicators of Toxicity 44
DNA Integrity and Sperm Damage 45

Main Conclusions 47

Recommendations 49

Glossary of Institutions and Organisations 50

References 52

Review of Chemical Toxicity to the Reproductive System, with Particular Reference to Developmental Toxicity

Introduction

Reproductive and developmental disorders are a significant cause of health detriment. It has been estimated that approximately 14-17% of couples desiring children encounter problems achieving pregnancy and where pregnancy is achieved, between 2-4% of newborns may have major birth defects [1-3]. These major malformations may be pronounced and require significant surgical or other intensive health care interventions or may even be lethal [3]. Clearly, they may be highly distressing for families and individuals affected. Within the context of the UK population this means that of approximately 600,000 live births each year [4], around 12,000-24,000 live births result in an infant with a congenital malformation. Of these malformations in live births, 720-1920 are thought to occur as a result of maternal illness, infection or chemical (including medicines, alcohol, drugs of abuse) exposures. The aetiology of 50-60% (6000-14400) of other congenital abnormalities is unknown [3]. The possibility that exposure to drugs or environmental chemicals may contribute to part of this figure cannot be excluded. Clearly, there is a need to maintain a contemporaneous knowledge of this subject in order that the HPA may provide authoritative advice on the reproductive effects of chemicals and to best identify where HPA resources should be used in this area to ultimately reduce the incidence of chemical related malformations.

Human embryonic and foetal development are highly complex events incorporating a multitude of signalling pathways and gene transcriptions to ensure correct cell differentiation, proliferation and patterning of the developing foetus. Inappropriate actions at any one of a number of stages may result in an abnormality, which may affect structure, function, and size or even prove lethal *in-utero* or *post-partum.* Maternal age is also a risk factor that has been associated with the incidence of certain congenital abnormalities. For example, the increased incidence of the chromosomal abnormality trisomy 21 (Down syndrome) in older mothers or the increased incidence of the non chromosomal defect gastroschisis (a fissure in the anterior abdominal wall, usually accompanied with projection of viscera) in the children of young mothers [5, 6].

The terms “teratogen” has been used to describe “an agent that if it is administered to pregnant mothers causes directly or indirectly, structural or functional abnormalities in the foetus or child after birth, which may not be apparent until later life” [7]. The term agent is used as a range of non-chemical exposures or disease states may also be teratogens and affect development such as ionising radiation or viral infections such as rubella.

Perhaps one of the starkest example of chemicals acting as teratogens was the thalidomide tragedy in the late 1950’s and early 1960’s where administration of this non-barbiturate sedative for morning sickness resulted in limb and cardiac defects in several thousand pregnancies [8, 9]. As a consequence of this tragedy, however, drug testing protocols were extensively revised to attempt to reduce the likelihood of a recurrence of such an event.

However, there remains little information currently available on the potential toxic effects to human pregnancy for the majority of chemicals used in industry or the environment. Recent initiatives seek to address this including the voluntary OECD

initiative based on Screening Information Data Sets (SIDS) and the extensive REACH proposals in the EU which will be mandatory [10].

The Toxicology Unit of the Chemical Hazards and Poisons Division was tasked to conduct a broad-based review of reproductive and developmental toxicology for the Health Protection Agency, and to identify possible areas for future work.

Section 1: Methods of Assessing Chemicals for Reproductive and Developmental Toxicity

It is beyond the remit and resources of the Agency to develop and validate novel techniques for use in chemical regulatory assessment. There are a number of initiatives underway in this and allied areas globally, such as the EC ReProTect initiative and new protocols will become available in due course [11].

Due to the complexity of mammalian biology, testing in living animals is currently key to the assessment of chemical hazards to human reproduction [12-14]. There are a number of *in-vitro* studies available which have been proposed as alternatives to animal studies or as pre-screens. These tests usually consider single aspects of reproduction such as effects on the embryo (embryotoxicity) and are, therefore, most suited for use as adjuncts to current testing frameworks, for example providing mechanistic information, rather than replacements.

Within the European Union there are around 30,000 commercially significant chemicals (i.e. produced in amounts greater than 1 ton *per annum*) that require evaluation and possibly further testing under the proposed EC regulations, REACH (Registration, Evaluation, Authorization of Chemicals) that is currently under review [10]. The types of data required for each chemical will be decided largely by its production volume, and therefore possible impact on the human population. It will not be practical to carry out the testing necessary to fill data gaps on all chemicals, and so some form of prioritisation will be necessary. *In-vitro* tests show particular promise in this regard and refinements in testing strategy by incorporating *in-vitro* methodologies may bring significant reduction in animal use.

The following chapter will outline the current *in-vivo* developmental and reproductive toxicity testing methods used within the Organisation for Economic Co-Operation and Development (OECD) test guidelines used in the regulatory approval of chemicals. *In-vitro* methods will then be considered, most notably those that are most promising and are suitable for consideration in a regulatory testing strategy. A number of other potential *in-vitro* methods are currently under development, and will be discussed briefly as will the EC ReProTect project.

In-Vivo Study Designs: OECD Guidelines on Assessment

At present, *in-vivo* testing in animals is necessary to investigate the effects of a chemical on the various stages of the reproductive cycle. There are basically two key studies required. One is the prenatal developmental toxicity study (historically known as a teratogenicity study) to investigate adverse effects on the developing foetus following in-utero exposure. The second is the one- or two-generation reproductive toxicity study to investigate effects on fertility and post-natal development.

Absence of data on reproductive toxicity is the most frequent 'data-gap' for chemicals in general use. Indeed adequate information on developmental toxicity and fertility is available only for a small proportion of the 30,000 or so chemicals produced in commercially significant amounts. This led to the development in the mid 1990s of more limited animal studies which provided 'screening' information on reproductive toxicity. These were specifically, OECD test guideline 421 (reproductive/developmental toxicity screening test) and the related test guideline 422 (combined repeated dose toxicity study). The latter was intended to be used when no adequate data were available on either repeated dose toxicity or reproductive toxicity. Although these tests use fewer animals and involve a more limited dosing period than the studies noted above, they still use appreciable numbers of animals. They have been widely used in the ongoing, voluntary programme on filling data groups for existing chemicals and, in particular, in the OECD high production volume Screening Information Data Sets (SIDS) Programme [15]. It needs to be recognised that these studies only provide 'screening information' and are intended to indicate when further investigation of reproductive effects is warranted. In particular, they provide only limited information on embryo/foetal toxicity and on post-natal manifestation of effects arising from pre-natal exposure or exposure via lactation.

Chemical testing for regulatory submission within the UK is usually conducted to guidelines set out by the OECD. Since their introduction in 1982, these harmonised guidelines have been an effective mechanism in reducing duplication of animal studies for regulatory purposes, as a study approved in one member state using the guidelines is acceptable in the regulatory authorities of all other OECD member states [16].

OECD guidelines require the use of the most relevant species in regulatory work and that the species and strains are those used most commonly in testing. Thus for developmental toxicity studies, the preferred rodent species is the rat and preferred non-rodent species is the rabbit. The use of other animal species must be justified, unless otherwise specified in the guideline and may be due to toxicokinetic differences between the preferred species and humans or to clarify ambiguous results obtained. The aim of these studies ideally is to derive No Observed Adverse Effect Levels (NOAELs) for risk assessments and an understanding of reproduction, parturition, lactation, and postnatal growth.

The key *in-vivo* studies required for developmental toxicity assessment are summarised in Table 1.

Table 1: Overview of Key In-Vivo Tests for Developmental and Reproductive Toxicity testing: OECD guidelines. (Adapted from Draft Guidance Document on Reproductive Toxicity Testing and Assessment, OECD Environment, Health and Safety Publications, Series on Testing and Assessment No 43, 2004 [17])

OECD Guidelines	Exposure Period	Endpoints in offspring
TG 414: Prenatal Developmental Toxicity Study	From implantation to one day before the day of scheduled kill	Death Resorptions Embryonic development Foetal growth Morphological variations and malformations
TG 415: One-Generation Reproduction Toxicity Study **TG 416:** Two-Generation Reproduction Toxicity Study	Continuous over 1, 2 or multiple generations	Growth, development and viability Histopathology of sex organs, brain and target organs Fertility Assessment of oestrus cyclicity and sperm quality (motility and morphology)
TG421: Reproduction/Developmental Toxicity Screening Test **TG422:** Combined Repeated Dose Toxicity Study with the Reproduction/Development Screening test	From 2 weeks prior to mating until Day 4 post natally	Fertility Pregnancy length and birth Foetal and pup growth and survival until Day 3
TG426: Developmental Neurotoxicity Study (DRAFT PROPOSAL FOR A NEW GUIDELINE, 2003)*	During Pregnancy and Lactation	Birth and pregnancy length Physical and functional maturation Behavioural changes due to central and peripheral nervous system effects Brain weights and neuropathology

**In 1995 an OECD expert working group recommended the development of an "OECD guideline on developmental neurotoxicity based on the existing EPA guideline". Following several consultation meetings, this draft was circulated for comments.*

More detailed information on each study, compiled from the respective OECD guidelines and OECD guidance notes, is provided below.

TG 414: Prenatal Developmental Toxicity Study (Updated Guideline, adopted 22nd January 2001)

This study guideline provides general information on the effects of prenatal exposure on the pregnant female (maternal toxicity) and the developing foetus (growth retardation, anatomical variations, teratogenicity and lethality).

At least 3 treatment groups and one control group should be used, with the control remaining untreated or receiving the same vehicle as the highest dose group. The highest dose groups should ideally cause toxicity (but not mortality) in the parents; the intermediate doses should cause minimal toxic effects and the lowest dose level ideally one that does not induce any observable effect in parents or offspring. All groups

should contain sufficient animals to yield about 20 females with implantation sites at necropsy.

Originally, the study considered the effects of exposure during organogenesis; with dosing conducted on Days 6-15 in the rodent and Days 6-18 in the rabbit. These periods are the most sensitive for inducing overt structural abnormalities. However, both the brain and gonads continue to develop beyond these days, and so the full potential for inducing structural and functional abnormalities cannot be assessed in these organs.

For this reason the updated guideline requires from implantation (Day 6 in rodent and rabbit) until one day before the dam is due to be killed. On the scheduled day, shortly before caesarean section, the females are killed and the uterine contents examined, and the foetuses evaluated for both soft tissue and skeletal muscle changes.

The total number of implantations may be assessed using TG 414: i.e. the number of living embryos, dead embryos and resorptions (number of embryos that die early and are re-assimilated). The degree of resorptions or the extent to which resorption has occurred (i.e. total, early and late resorptions) may be used to determine the time of death of the embryo during pregnancy. Where dosing is started before Day 6, pre-implantation losses may be also be determined.

Functional deficits and other post natal effects are not considered in this study guideline, but information on such aspects may be obtained from the reproductive toxicity studies considered below.

See Table 1 for summary.

TG 415: One-Generation Reproduction Toxicity Study
(Original Guideline, adopted 26th May 1983)

This study guideline provides general information concerning the effects of a test substance on male and female reproductive performance in the parent (P) generation, such as gonadal function, oestrous cycle, mating behaviour, conception, parturition, lactation, and weaning. The study may also provide preliminary information about the developmental toxic effects of the test substance in the F_1 generation, including neonatal morbidity, mortality, teratogenesis, behaviours (in rearing) and serve as a guide for other tests. Rats and mice are the preferred species for this study.

At least 3 treatment groups and one control group should be used, with the control remaining untreated or receiving the same vehicle as the highest dose group. The highest dose groups should ideally cause toxicity (but not mortality) in the parents; the intermediate doses should cause minimal toxic effects attributable to the test item and the lowest dose level ideally one that does not induce any observable effect in parents or offspring. All groups should contain sufficient animals to yield about 20 pregnant females at or near term. Clearly, this may be a problem when testing substances that cause early sterility, although such adverse effects on fertility would be demonstrated.

The testing procedure requires males and females to be dosed for a sufficient duration before mating such that effects on spermatogenesis and oestrous would be seen. This equates to one spermatogenic cycle (56 and 70 days in the mouse and rat, respectively) and at least 2 oestrous cycles. The animals are then mated, and are dosed throughout this period. On completion of mating, the females are dosed throughout gestation and for the duration of the nursing period up to weaning of the F_1.

Bodyweight is an important factor and may be a sensitive indication of developmental toxicity, indeed it may be the only indication.

See Table 1 for summary.

TG 416: Two-Generation Reproduction Toxicity Study
(Updated Guideline, adopted 22nd January 2001)

This study is conducted in a similar manner to the above, but with a number of additional investigations to provide a more comprehensive assessment of effects on reproductive function, specifically effects on the second generation.

As well as studying growth and development of the F_1 generation, the guideline aims to assess the integrity and performance of the male and female reproductive systems as well as growth and development of the F_2 generation. The study also provides information on the effects of the test substance on neonatal morbidity, mortality and preliminary data on prenatal and post natal developmental toxicity.

For additional information on developmental toxicity or functional deficit, additional study segments can be incorporated using guidelines for developmental toxicity and/or developmental neurotoxicity. These specific endpoints could also be considered in separate studies using the appropriate guidelines.

The testing procedure requires males and females of P and the subsequent F_1 to be dosed for a sufficient duration before mating such that effects on spermatogenesis and oestrous would be seen. This equates to at least one spermatogenic cycle (56 and 70 days in the mouse and rat, respectively) and several oestrous cycles. The animals are then mated, and are dosed throughout this period. On completion of mating, the females are dosed throughout gestation and for the duration of the nursing period up to weaning of the F_1 or F_2, as appropriate.

Males of P and F_1 are killed after mating, and effects on sperm are determined using a number of parameters (mortality and morphology) and in tissue preparation and detailed histopathology of testes and epididymides. Extensive histopathology conducted on the female reproductive system is also conducted.

Additionally developmental milestones such as optional behavioural parameters and histopathology of brain and identified target organs are included.

See Table 1 for summary.

TG 421: Reproduction/Developmental Toxicity Screening Test
(Original Guideline, adopted 27th July 1995)

This guideline is essentially an abbreviated version of TG 415 using smaller numbers of animals and reduced dosing periods, and may be used at an early stage in toxicological assessment especially when large numbers of compounds may require testing. It should help in identifying those substances of most concern that warrant more detailed investigation of reproductive effects. The study will generate limited information on the gonadal function of mating behaviours, conception, development of conceptus and parturition, which may be useful in determining additional or specific assessments.

The rat is the preferred species, and several groups containing at least 10 males and 10 females are dosed at least 2 weeks before mating and during a mating period of up to 14 days. Dosing is continued in the females until Day 4 *post partum* and males are killed after a minimum dosing period of 28 days.

The duration of exposure is clearly shorter than the 70 day spermatogenic cycle in the rat, and so extensive histopathological examination of the testes is conducted.

The total duration of the study is approximately 54 days, assuming 14 days premating up to 14 days mating, 22 days gestation and 4 days lactation before kill.

See Table 1 for summary.

TG 422: Combined Repeated Dose Toxicity Study with the Reproduction/Developmental Toxicity Screening Test (Original Guideline, adopted 22nd March 1996)

This guideline describes the procedure for a study in which a standard repeat dose toxicology test (c.f OECD TG 407) is combined with a reduced one generation study and is useful in situations where large numbers of compounds require testing and there is insufficient information available on both repeated and reproductive toxicity e.g. OECD SIDS programme. One TG 422 test may substitute conducting 2 separate studies; namely TG 421 and the repeat dose study of 28 days dosing under guideline TG 407.

The experimental procedure is similar to that described previously, but includes clinical pathology of males and females and options to incorporate functional observations.

See Table 1 for summary.

TG 426: Developmental Neurotoxicity Study Draft New Guideline (September 2003)
The OECD guideline for this investigation is still being developed

Developmental neurotoxicity testing is conducted to further characterise neurological effects observed in other studies, and should normally be considered if the substance has been shown to cause neuropathology or neurotoxicity in adults, to be hormonally active *in-vivo* or to cause other types of toxicity suggestive of nervous system involvement at a developmental stage or the exposure scenario warranted such intensive testing.

This study would usually be conducted at a late stage in the testing strategy. The observations and measurements could also be included in other study designs e.g. in a two generation reproduction study (TG 416).

The rat is the preferred species for this study, and twenty litters per dose level are recommended. Pregnant females are usually dosed daily from implantation (Gestation Day 6) throughout lactation. This allows pups to be exposed during both pre and post natal neurological development.

Offspring are randomly selected from within litters for neurotoxicological evaluations. These include observations to detect gross neurological and behavioural abnormalities, including sexual maturation, physical development, reflex ontogeny (e.g. negative geotaxis), motor activity, motor and sensory function, and learning and memory (e.g. simple mazes such as the Morris water maze or T-maze). The evaluation of brain weights and neuropathology during post natal development and adulthood is also included and is usually conducted in a number of pups from each group at Postnatal Day 22 and at study termination on Postnatal Day 60.

Assessments are conducted across 3 main age periods: Preweaning (before Postnatal Day 21, adolescence (Postnatal Day 21-59) and young adults (Postnatal Days 60-75). Maternal females are killed and discarded after weaning on Postnatal Day 21.

The measurements and observations given in the guideline may also be incorporated into a one or two generation reproduction toxicity study.

See Table 1 for summary.

In-Vitro Study Designs

Recognition of the importance of reproductive toxicity, and the major gap in the knowledge base for most chemicals in this area, has led to much interest in developing *in-vitro* methods that can be used in a screening mode to prioritise compounds for investigation using animal experiments. A number of innovative strategies have been developed and include the application of cell or embryo culture techniques.

However, investigation of the effects of a chemical on all aspects of reproductive function covers a multitude of endpoints and presents difficult challenges with respect to the development of alternatives. Numerous *in-vitro* tests would be needed if an attempt was made to cover all endpoints (for example, studies on the use of Sertoli cell lines, sperm mutations, morphology and penetration assays have been suggested to investigate effects on male fertility). This has led to initial efforts being mainly concentrated on one particular area, namely screening for compounds that may induce marked embryotoxicity (malformations) following *in-utero* exposure. This is because such teratogenic chemicals are of particular concern.

In-Vitro Assays for Embryotoxicity

There are many embryotoxicity testing systems which have been considered for regulatory applications. These include a number of lower-order assays including Frog Embryo Teratogenesis Assay: Xenopus (FETAX) and Zebra fish oocytes. In the FETAX assay, early Xenopus Laevis (African Clawed frog) embryos are exposed at the notochord stage to determine toxicity or teratogenicity in the developing CNS. Exposure in the late premetamorphic larvae can be used to determine toxicity in the developing skeletal system of the tadpole as well as functional assessment of swimming ability [18, 19]. The assay, while being low-cost and rapid, is limited by the need for aqueous solubility of test substances, the lack of validation and by the small number of laboratories that have used the system [13]. In the Zebrafish assay, fertilised eggs are exposed for 48 h, and multiple endpoints assessed[1] [20]. Concern over species differences favours the use of vertebrate systems for screening for teratogenicity and this use of lower-order species may be therefore more suited to ecotoxicology monitoring [21].

Avian chick embryos have been used as models in developmental biology, but have not seen wide use in embryotoxicity testing [13]. One general problem noted in the results of the chick embryotoxicity screening test (CHEST) being the inability to distinguish general toxicity from specific developmental effects [21].

A number of promising tests have been subject to validation studies by the EC's European Centre for the Validation of Alternative Methods (ECVAM). These tests are not considered to be replacements for current animal methods, but could provide suitable means for reducing and/or refining the use of animal procedure in the context of testing strategies e.g. by prioritisation of compounds for more detailed evaluation.

The following three tests have been the focus of most attention: - the embryonic stem cell test (EST); the micromass test; and whole embryo cultures (Table 2).

[1] Endpoints assessed include coagulation, development of blastula gastrulation, termination of gastrulation, development of somites, extension of tail, development of eyes, heart beat, circulation, pigmentation and oedema.

Table 2: Overview of Key In-Vitro Tests for Developmental and Reproductive Toxicity Testing

Study	Test System	Endpoints
Embryonic Stem Cell Test (EST)	Two permanent mouse cell lines: D3 embryonic stem cells and 3T3 fibroblast cells	Inhibition of ES cell differentiation into cardiac myoblasts Inhibition of D3 proliferation Inhibition of 3T3 proliferation
The Micromass (MM) test (Method of Brown)	Limb buds isolated from rat embryos	Inhibition of foci of differentiating chondrocytes
Postimplantation rat whole embryo culture (WEC) (method of Piersma)	Rat embryos (1-5 somites)	Morphology: malformations, specific growth retardations/failures to differentiate Functionality and viabilities: heart beat, yolk sac and allantoic circulation Embryo growth; yolk sac diameter, crown rump length, head length

These studies are considered in more detail below.

Embryonic Stem Cell Test (EST)

The assay is based on the potential of embryonic stem cells to differentiate in culture and is the most promising approach. Two permanent mouse cell lines are used: D3 mouse embryonic stem cells to represent embryonic tissue and 3T3 fibroblasts to represent adult tissue. The mouse embryonic stem cells can be maintained in an undifferentiated state in the presence of cytokine leukaemia inhibiting factor (LIF). When released from the undifferentiated state, the stem cells will form embryonic bodies and differentiate under appropriate conditions into major embryonic tissue. Cytotoxicity data show that the embryonic stem cells are more sensitive to toxic agents than adult cells. The inhibition of differentiation of the stem cells and the inhibition of growth of D3 stem cells and 3T3 cells (as a measure of cytotoxicity to the embryonic and adult cells respectively) are the three key endpoints in this test. An algorithm is applied to the values obtained and a determination of *in-vivo* embryotoxicity potential made which classifies substances into 3 categories: strong, weak or non-embryotoxic [22]. This prediction model is based on *in-vivo* data derived from a series of chemicals, and so acts as a reference for the *in-vitro* findings.

In validation studies to date, the mouse embryonic stem cell assay has involved measuring the development of cardiomyocytes from undifferentiated stem cells, together with cytotoxicity [23]. Since many of the possible mechanisms leading to developmental defects[1] are 'general' biological mechanisms, the embryonic stem cell assay is able to identify chemicals that interact with non-target cell specific events, as well as with chemicals that act specifically on cardiomyocyte differentiation. Cardiomyocytes are selected as the endpoint for differentiation as they can be easily distinguished within the culture of differentiating cells, and because the heart is the first organ that develops in organogenesis [24].

[1]e.g. mitotic interference, altered membrane function/signal transduction, altered energy sources, enzyme inhibition, altered nucleic acid synthesis, induction of mutations etc

An early protocol for this test [25] was refined in an ECVAM pre-validation study [26] and then subjected to a formal international validation study [22]. Based on the results of the latter, the ECVAM Scientific Advisory Committee (ESAC) agreed that the assay was highly reproducible and shared good correlation with the *in-vivo* data. The test was applicable to a diverse group of chemicals with different embryotoxic potential. ESAC concluded that the mouse embryonic stem cell assay was a scientifically validated test which was ready for consideration for regulatory purpose [27].

However, as a general comprehensive screen for embryotoxicity, it was recognised that there was a need to further develop the test to allow measurement of target tissues other than the heart; specifically, neural and skeletal systems. Furthermore, there is currently no optimised approach for the incorporation of an exogenous metabolic activation system in this assay such. These limitations are being addressed in the EC's ReProTect Project [11], discussed later.

See Table 2 for summary.

Micromass Assay (MM)

This approach is based on the micromass culture technique of mouse limb buds by Umansky [28]. Rat embryo midbrain or limb buds are used to investigate the ability of test compound to inhibit cell differentiation and growth as measured by inhibition of foci formation of chondrocytes or neurones [29]. This technique therefore reproduces early mammalian cartilage histogenesis, a key step in skeletal development. Pregnant rats are killed on Day 14 of gestation, the uteri excised and the embryos removed from the implantation sites. Embryos with 45±5 somites are selected, and the limb buds removed with a single cut. A single cell suspension is prepared using trypsin, and adjusted to approximately 2 x 10^7 cells/mL using dilutions of appropriate volumes of medium. From this suspension, drop cultures are prepared in 96 well plates and incubated with a range of concentrations of test chemical. Cell differentiation is determined by Alcian blue staining (cartilage-specific proteoglycan stain) and cell viability and growth assessed by neutral red uptake and counts of both foci number and cell number [30].

The limb bud micromass assay was included in the ECVAM international validation study on *in-vitro* embryotoxicity assays. The method performed more poorly than the mouse embryonic stem cell assay. Although the data were reproducible, the method only detected strongly embryotoxic compounds [31].

See Table 2 for summary.

Post-Implantation Rat Whole Embryo Cultures (WEC)

In this assay rat embryos with 1-5 somites (obtained at gestational day 10) are cultured for 48 hours in the presence or absence of the test compound. Major aspects of organogenesis occur during this period (heart development, closure of neural tube, development of eye and ear, bronchial bars, and limb buds). The embryos are then assessed in the order in which they were placed into culture, and scored with regard to morphology (gross examination), functionality (heart beat, yolk sac and allantoic sac circulation) and embryo growth.

This method has been widely used to investigate the mechanism of action of teratogens. From the mid 1990s attempts were made to optimise this method as an

approach for screening for teratogenic agents [32]. The method was the third assay included in the ECVAM international validation study on embryotoxicity assays. The results obtained were comparable to those obtained using the mouse embryonic stem cell assay. The ESAC concluded that the assay was reproducible and that the correlation between *in-vitro* and *in-vivo* results was good. It was felt that the assay was a scientifically validated method that was ready for use for regulatory purposes.

Whole embryo culture systems have the major advantage of assessing embryogenesis in its full complexity from cellular proliferation to differentiation and to pattern formation [14]. Furthermore, embryo culture allows accurate determination of the doses to which embryos are exposed to, something not so readily obtainable from *in-vivo* study types [33].

However, from an animal use perspective, this alternative has the disadvantage of using embryos obtained from female rats at Day 10 of gestation. It is likely that its main use will continue to be used in mechanistic studies rather than as a screening approach for detecting compounds with the potential to produce adverse effects on the developing offspring.

A further disadvantage of this system is the limited period of embryogenesis that is undertaken in the commonly used culture system as some chemicals may exert their major toxicological effect late in gestation [33]. For this reason, as with many other *in-vitro* studies, they are used in association with other studies to allow complete assessment of development.

See Table 2 for summary.

Conclusion regarding EST, MM and WEC protocols

Given the less robust nature of the micromass assay and the need to use animals in the whole embryo culture models; future work in developing *in-vitro* assays for screening chemicals to detect those with embryotoxic potential is likely to concentrate on the use of stem cell assays.

Novel Strategies for *In-Vitro* Assessment

There are a number of technologies which may see increasing use in reproductive and developmental toxicity assessment. These include computer modelling strategies, modifications to existing systems, Real–Time Quantitative Polymerase Chain Reaction (RT-PCR), and DNA micro arrays. Outlines of those not covered earlier are given below.

Quantitative Structure Affinity Relationships (QSAR)

These models are based on the premise that biological activity is implicit from chemical structure and aims to formalise the relationship between the two [34]. The outcomes of exposure to a chemical could then be predicted from existing knowledge of the functional groups within the chemical, or from structurally similar compound of the same or similar class.

There are a number of models available including MULTICASE and TOPKAT systems [35], but none have been validated as providing sound data regarding reproductive toxicity. Modelling is limited in developmental toxicity assessments by the plethora of possible mechanisms of toxic action and endpoints involved [11, 36].

Evaluation by QSAR is a rapid technique and within the requirement of REACH to test large numbers of chemicals, these tools offer some promise as far as screening by providing mechanistic hypothesis that may guide future testing.

DNA Microarrays

Differential gene expression assays using microarrays have been suggested as a growth area for studying developmental toxicity [14]. An example of their potential is Naciff et al (2002), who examined the gene expression profiles in the ovaries of rats exposed *in utero* to 3 chemicals with estrogenic activity [37]. Ovaries of litter mates were pooled, the RNA isolated and converted to double-stranded complimentary DNA (cDNA). This was subsequently labelled and hybridised to oligonucleotide microarrays of rat genome for 16 h. The non-hybridised material was removed and the changes in expression determined from fluorescent imaging. The different expression profiles for each chemical indicated that oestrogenic activity occurred by different mechanisms. A high throughput screening protocol could potentially compare expression profiles with existing data from known teratogens or with structurally similar compounds. A review of DNA microarray technology and its potential applications in toxicology is presented in Rockett and Dix, 2000 [38].

European Community ReProTect Project; Future Work on the Validation of Alternative Methods

The EC has initiated a large, five year program referred to as ReProTect [11]. The overall aim is the integration of existing *in-vitro* models and newly developed models into a test strategy that will provide detailed information on the hazard of chemicals to the mammalian reproductive cycle. Key aspects of this programme are:

A) Fertility

Work relating to method optimisation and testing of reference compounds in the areas listed below.

Male Fertility

- Detection of chemical effects on mature spermatozoa using high throughput screening methods (sperm mutations using Computer Assisted Sperm Analysis; CASA)
- DNA damage in mature spermatozoa using the Comet assay
- Testosterone production by a genetically engineered Leydig cell line
- Development of Sertoli cell lines for use in generation of data relevant to sperm maturation and possibly modelling blood testis barrier

Female Fertility

- Development of a test for *in-vitro* maturation of bovine oocytes
- Development of a test for *in-vitro* fertilisation of bovine oocytes
- Development of i*n-vitro* cultures of bovine pre-implantation embryos to investigate effects on early embryo development
- Development of *in-vitro* immortalised granulosa cell cultures and measurement of steroidogenesis in mouse follicle cultures and its granulosa cultures

B) Implantation

Work in this area will start around 2006/2007. There have been two workshops held to define the problems relating to uterine function and the implantation process and these will be used to identify a specific work programme.

C) Prenatal Development (Embryotoxicity)

Effort will concentrate on widening the applicability of the mouse embryonic stem cell assay as an *in-vitro* test for teratogenicity. It has been validated for measuring differentiation into cardiac myoblasts. This will be extended to neural cells (for developmental neurotoxicity) and chondrocytes (for skeletal effects).

An appropriate exogenous metabolic activation system will also be developed for use in this assay.

The use of a human embryonic stem cell line (H1) will be evaluated.

Conclusion on Testing Strategies in Developmental Toxicity

Given the complexity of the embryonic system, it is clear that no single alternative will be able to replace *in-vivo* testing [14]. Even the best currently available *in-vitro* techniques are limited by biology, since none of the tests predict functional abnormalities induced by chemicals [13]. However, *in-vitro* methods are potentially rapid and lower-cost screening tools for the evaluation of chemicals for reproductive toxicity that may also reduce the number of animals required in assessing human health risks. They clearly will contribute to the policy aim of the "3R's" i.e. the refinement, reduction and replacement of animals in experimentation. These methods usually consider short durations of the development process or specific aspects and so testing strategies using a number of screens will be required. The use of human metabolic activation/detoxification systems may be considered in such a package [24]. The use of human cell lines is still problematic, for a variety of ethical and legal reasons, but may be an area that could see expansion in toxicity screening.

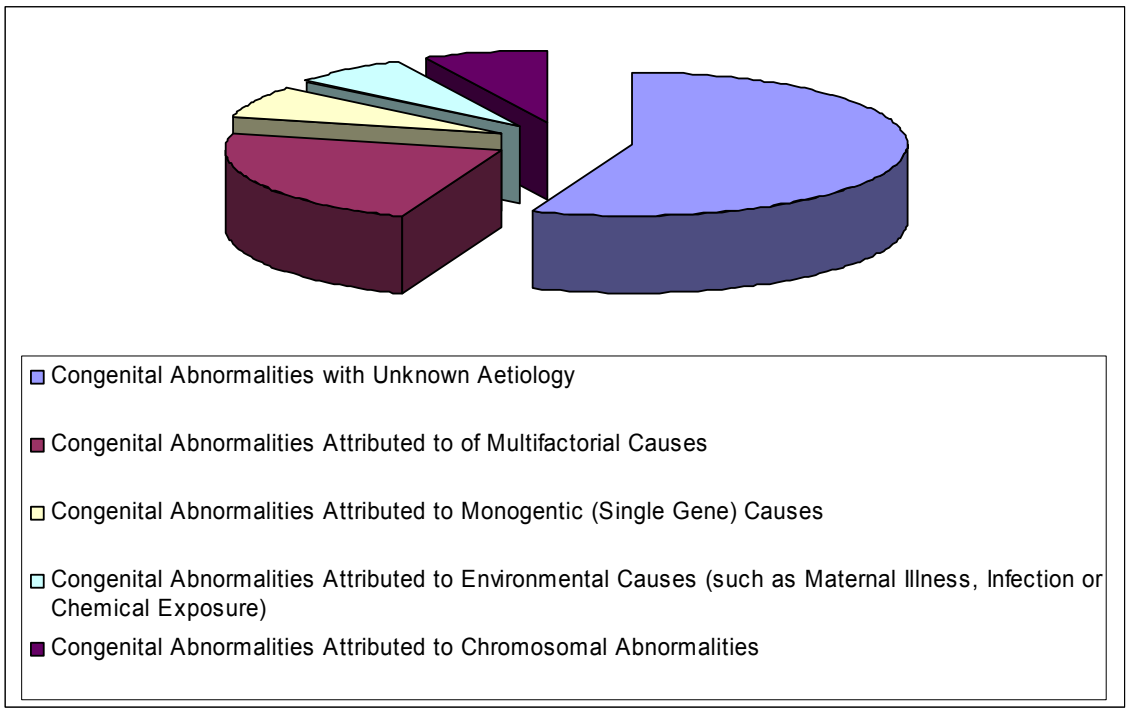
It is likely that most progress will be made in pre-screening of early embryotoxicity through the use of combined strategies. For applications such as QSAR and DNA arrays, a sound understanding of mechanisms associated with teratogenicity is required before these technologies can be fully exploited within a regulatory context.

Section 2: Chemical Teratogenesis

Congenital malformations are a considerable public health issue; although the majority of neonates are normal at birth. Registries of congenital anomalies report that 2-3% of neonates may be born with a single major malformation and a further 1% with multiple major malformations [3]. The figure for all major malformation may rise to 5% by the time the child is 5 years old, when other functional and behaviour deficits may become apparent [7]. These major malformations may be pronounced and require significant surgical or other intensive healthcare interventions or may be even lethal [3]. Clearly, such congenital malformations may be highly distressing for families and individuals affected. It should also be noted that in terms of total abnormalities these figures are conservative, as spontaneous abortions may occur where malformations are pronounced, especially in the first trimester of pregnancy [3].

In terms of causation, figures in the recent comprehensive EUROCAT special report [3] indicate that the aetiology of congenital malformations is unknown in as many as 50-60% of cases. Of the remainder, 20-25% are considered to be multifactorial in origin, 6-8-% as monogenetic (single gene) and 6-8% to be due to chromosomal abnormalities [3]. Around a further 6-8% of all developmental abnormalities have been attributed to environmental risk factors such as maternal illness, infection and chemicals (including medicines, alcohol, drugs of abuse) [3]. These values are represented graphically in Figure 1.

Figure 1: Graphic Representation of the Attributed Aetiology of Congenital Abnormalities (Median Values of Ranges Employed)



It is probable that an increased awareness of human genetics will identify the basis of some of the unknown malformations and possibly identify strategies for reducing their occurrence. In terms of mechanisms of chemical teratogenesis, the specific molecular

target for even the more thoroughly researched teratogens (such as ethanol and thalidomide) have not been identified with certainty [39, 40]. Several syndromes are associated with specific chemical exposures such as the limb and cardiac effects from thalidomide exposure or the neural-tube defects associated with classic anti-epilepsy drugs such as sodium valproate, but the likely outcomes in humans following exposure to most teratogens is not well understood or readily predictable. The situation is complicated further, as similar teratogenic outcomes have been noted from exposure to structurally diverse compounds in animals, suggesting a number of factors or pathways may be involved. A wide range of outcomes in teratogenesis have been described and are outlined below:

Table 5: Possible Teratogenic Outcomes (Adapted from McElhatton, 1999 [7])

Possible Teratogenic Outcomes
Induction of chromosomal abnormalities
Prevent implantation of the conceptus
Cause resorption of the early embryo
Cause structural malformations e.g. organ or limb defects
Changes in intrauterine growth leading to retardation
Cause embryo or foetal death
Cause functional impairment in the neonate e.g. deafness
Cause behavioural abnormalities
Cause mental retardation
Cause changes that may lead to increased incidence of neoplasia e.g. Diethylstilbestrol

The extent of our understanding is also limited by practical considerations: it is often sufficient to identify a teratogen within a regulatory framework and not necessarily to explore the way in which the teratogenesis is affected. With our growing understanding of human development through genomic and other technologies, we may be better able to determine the interactions and factors that may contribute to teratogenesis. The multifactorial nature of teratogenesis requires that chemicals are evaluated in the context of a large number of other contributory factors [41], the interplay of which is considered in Figure 3.

This section will consider some of the basic knowledge relating to teratogenesis. Definitions will be presented, principles outlined and a number of chemical interactions that could be potentially teratogenic discussed. While the HPA concerns relate to environmental chemicals, reference will be made to several pharmaceuticals, for illustration, where certain mechanistic interactions are better understood.

Teratogens and Teratogenesis

A human teratogen may be considered as "an agent that if it is administered to pregnant mothers causes directly or indirectly, structural or functional abnormalities in the foetus or child after birth, which may not be apparent until later life" [7]. The term agent is used as a range of non-chemical exposures or disease states may also be teratogens and examples are provide below (Table 3) and also in the EUROCAT Review [3]).

Table 3: Examples of Factors with Known or Possible risks of Harm to the Unborn Child [3, 42]

Teratogenic Factor
Ionising Radiation
Infections:
Cytomegalovirus (CMV)
Rubella virus
Toxoplasmosis
Varicella virus
Metabolic Imbalances or Disease States:
Alcoholism
Diabetes
Folic acid deficiency
Hyperthermia
Iodine deficiency
Phenylketonuria
Diagnostic (Physical Injury):
Amniocentesis (early)
Chorionic villus sampling (before day 60)
Lifestyle Factors:
Drugs of abuse, e.g. solvents such as toluene and stimulants such as cocaine or amphetamine
Excessive alcohol consumption
Smoking
Medication:[†]
ACE inhibitors and angiotensin –II receptor antagonists
Aminoglycosides
Anti-epileptics
β-adrenoceptor antagonists
Cytotoxic drugs
Hormones; androgenic or oestrogenic e.g. Diethylstilbestrol
Retinoic Acid
Tetracyclines
Thalidomide
Warfarin/coumarins

[†] ***N.B.*** *Absence of a drug from this table does not imply safety. Pharmaceuticals are not the key focus of this document, and therefore only a selection is provided within this listing. Healthcare professionals should refer to the NTIS entries on the HPA-commissioned Toxbase® website, or other specialist references, for fuller listings and advice.*

It must be stressed, however, that as with toxicology as a whole, teratogenicity is a property of an exposure which involves not only the physical and chemical nature of the agent but also: the dose, the route of exposure, the biological susceptibility of the mother and embryo or foetus and, in this case, the gestational timing of exposure as well as [42]. The factors are considered together in general principles of teratology. There are 6 general principles of teratology as established by Wilson; these important generalisations, put forward first in 1959 [43] and amended in 1973 [44], are still considered valid [45] and are presented in Table 4.

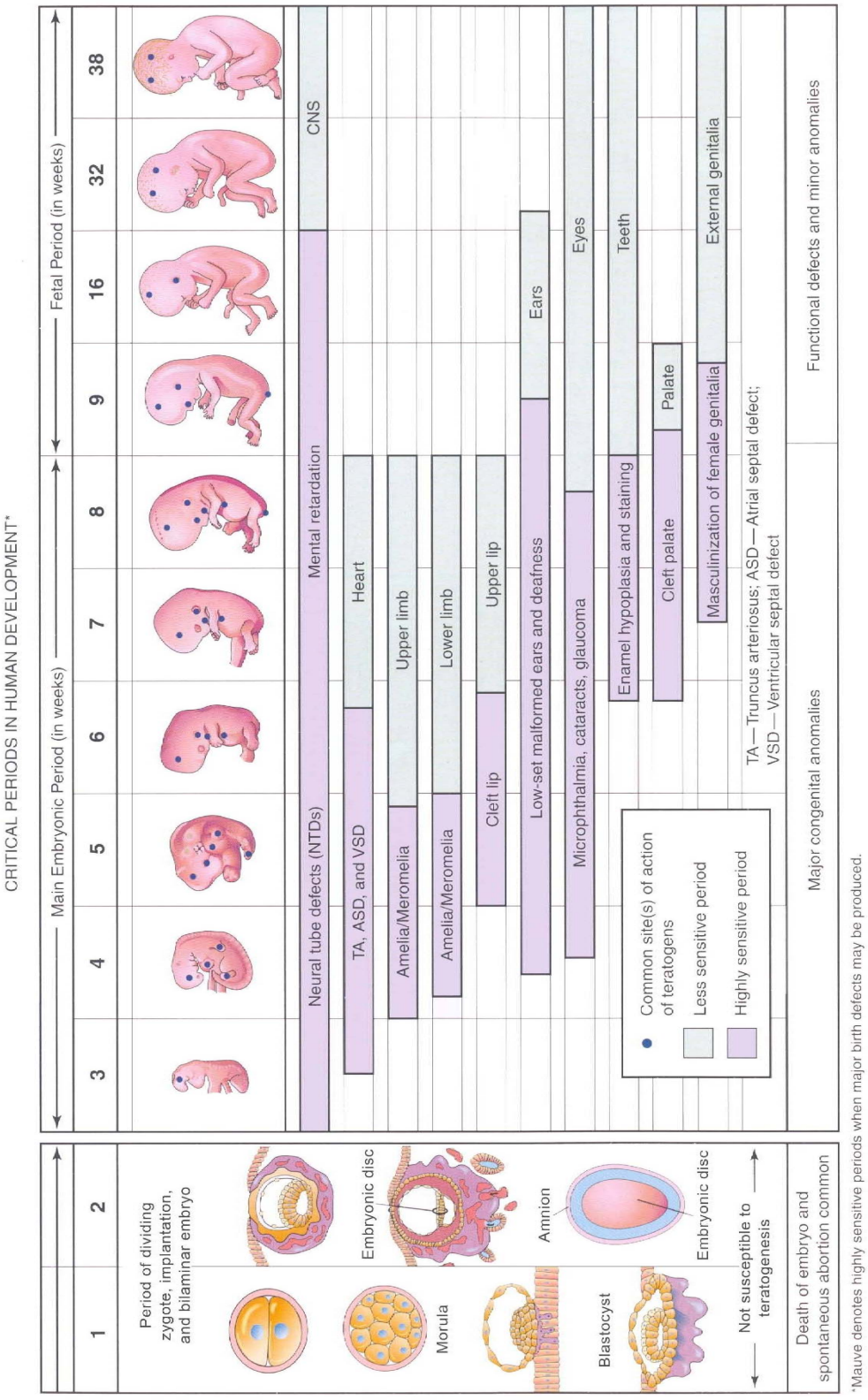
Table 4: Principles of Teratology, Adapted from Wilson (1973) [44]

#	Principles of Teratology
I	Susceptibility depends on the genotype of the conceptus and a manner in which this interacts with adverse environmental factors
II	Susceptibility to teratogenesis varies with the developmental stage at the time of exposure to an adverse influence.
III	Teratogenic agents act in specific ways (mechanisms) on developing cells and tissues to initiate sequences of abnormal developmental events (pathogenesis)
IV	The access of adverse influences to developing tissues depends on the nature of the influence (agent)
V	The four manifestations of deviant development are death, malformation, growth retardations and functional deficit
VI	Manifestations of deviant development increase in frequency and degree as dosage increases, from the no-effect level to the totally lethal level

The concept of especially susceptible periods within human development is of fundamental importance when considering congenital abnormalities and is illustrated in Figure 2. In terms of outcomes, it is perhaps unsurprising that structural malformations are a key focus of developmental abnormality research and it was almost, in the past, dogma that all these affects arise during the period of organogenesis, typically the time from appearance of the neural plate to complete palatal closure i.e. approximately Days 18-58 [41]. However, it is important to note, as in Figure 2, that development of reproductive organs occurs late in pregnancy and that mental development is an ongoing process. Indeed the recognition of the importance of later stages has led to the requirement when testing in animals for developmental toxins that dosing occurs from implantation to the end of pregnancy, rather than just the period of organogenesis.

An additional consideration is that not all structural defects noted at birth are caused at an early stage of developmental or are the result of genetic dysfunction or direct chemical or infectious teratogenesis on the foetus itself: a small number of defects may not be malformations *per se*, but deformations arising from amniotic bands [46]. These may arise spontaneously from tears in the amnion leading to fibrous string formations that may entangle the foetus [47]. While uncommon, this effect may cause minor deformities, amputations or death in the most severe cases.

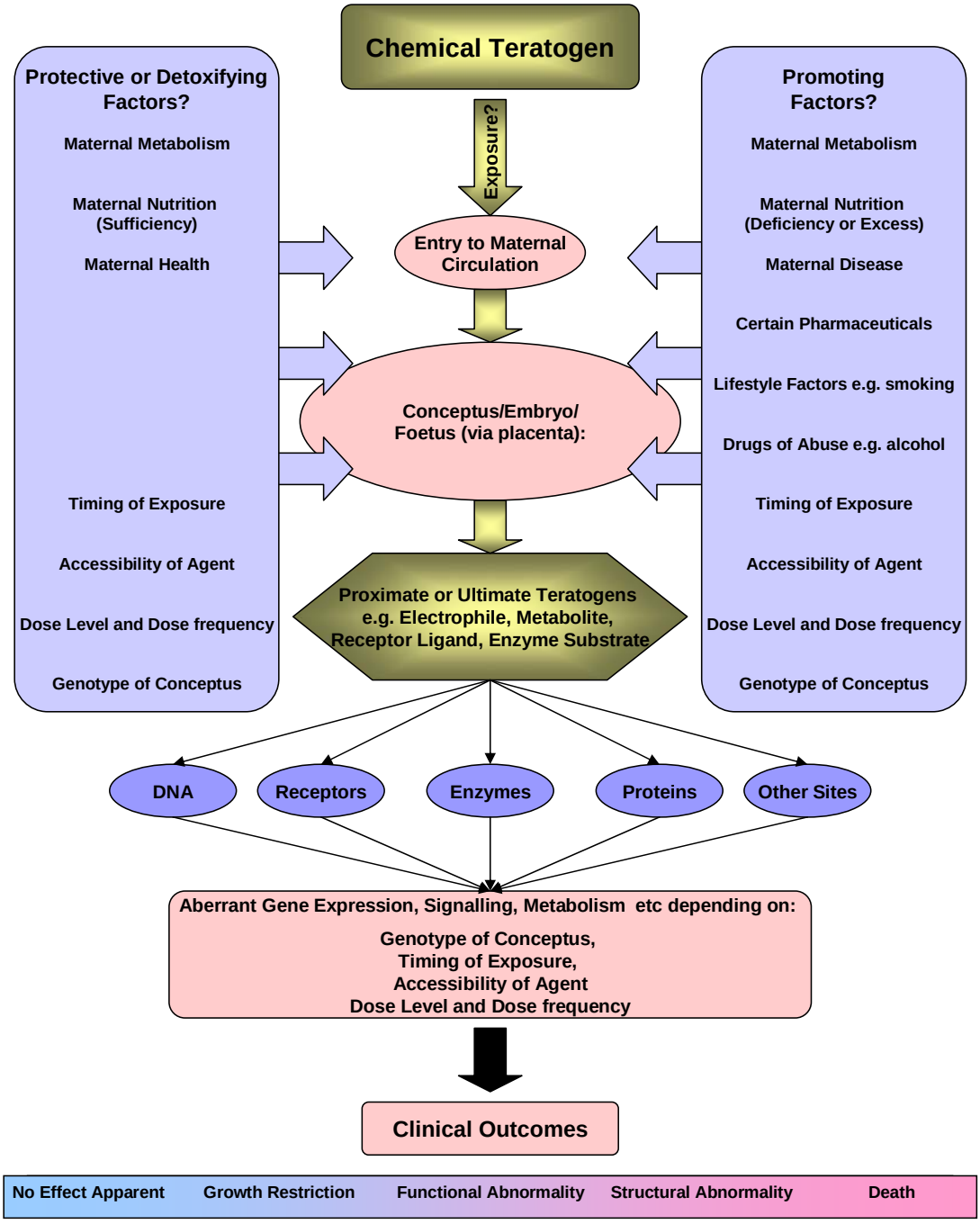
Figure 2: Crucial Periods in Human Prenatal Development. (Reprinted from: The Developing Human: Clinically Oriented Embryology. 7th Edition. Moore & Persaud. Crucial Periods in Development; Page No 520. Copyright (2003): with permission from Elsevier [48].



Dots on the developing foetus show common sites of action of teratogens. Horizontal bars indicate fetal development during a highly sensitive period (purple) and a less sensitive period (green)

A schematic incorporating the range of factors and principles in chemical teratogenesis discussed is presented in Figure 3. The schematic describes a putative exposure which could lead to a spectrum of birth outcomes, dependent on the principles described above in Table 4 such as the dose and timing of exposure. It is noteworthy that multiple external factors such as maternal health, dietary status are in place in the absence of exposure to a chemical teratogen; the majority of congenital abnormalities are considered not to be related to chemical or indeed environmental exposures [3].

Figure 3: Schematic Representation of Chemical Teratogenesis



Mechanisms in Chemical Teratogenesis

The question of mechanisms in teratogenesis is complex. When Wilson formalised principles in teratology over 30 years ago, he noted the difficulty arising in identifying the first event in teratogenesis which could be at a sub-cellular or molecular level [44]. There are a number of plausible modes of action that have been proposed, including the impact of reactive oxygen species stemming from the metabolism of certain compounds. However, the specific mechanism i.e. the target, action and progression that produce for example, a functional deficit is, broadly speaking, not well defined in the literature.

As described previously, outcomes from teratogens may vary with the time of exposure, the discreet target or a combination of targets and mechanisms and well as a range of possible external factors which may also be involved (Figure 3). Consequently, this section will focus on a number of general mechanisms implicated in teratogenesis quoting examples where possible to illustrate such mechanism. However, the specific mechanism of action of most teratogens is largely unknown.

Direct DNA Damage: Mutations and Chromosomal Damage

Chemically-induced changes in nucleotide sequence may result in harmful mutations. These may be hereditable, as in the case of germ cell mutations, or non-heritable as in the case of somatic cell mutations. The outcome in somatic cells may be one of neoplasia or aberrant development. Early (pre-implantation) exposure to mutagens such as methylnitrosourea may produce death of the conceptus and a wide range of abnormalities in animal embryo studies [49]. Another example of a mutagen that can produce abnormalities is the anti-neoplastic agent, cyclophosphamide. The parent compound is not teratogenic, but is metabolised to phosphoramide mustard which may cause chromosomal damage resulting in genetically defined morphological changes and apoptotic and necrotic cell death [39].

While developmental abnormalities may arise from exposure to a range of anti-neoplastic agents, normal birth outcomes have been recorded in approximately 83-90% even after first trimester exposures [41, 50, 51]. This highlights the multifactorial nature of teratogenesis even when potent cytotoxic agents are involved at high clinical dose levels. Reasons for the difference in outcome could possible relate to the extent of foetal DNA repair processes or that embryo and foetus are metabolically incompetent which may limit some aspects of bioactivation.

While there is cause for concern relating to human exposure to mutagens, it is important to note that not all mutagens are teratogens and that not all teratogens are mutagens, as shown in section 3. It is also worth noting that diethylstilbestrol is currently the only defined human transplacental chemical carcinogen.

Diethylstilbestrol (DES) has been shown to be a mutagen and has been shown to covalently bind DNA [52], although the exact process of transplacental carcinogenesis is not known [39]. One postulated mechanism is that an electrophilic metabolite of DES acts as a tumour initiator, causing damage in the developing vagina and cervix *in-utero*, with oestrogens at the menarche leading to tumour promotion [41].

DES is a potent non-steroidal oestrogen, and was heavily prescribed from the late 1940s-1970s to women with high risk pregnancies, the intention being to reduce spontaneous abortions and other complications [53]. An increase in the prevalence of

a rare vaginal cancer (clear cell adenocarcinoma) as well as genital abnormalities in young girls and women whose mothers had taken diethylstilbestrol to support their pregnancy has been well documented [54-56]. A slightly increased incidence of testicular cancer was noted in males exposed *in utero* in one study, though larger studies with increased statistical power would be required to confirm this effect; the incidence of all cancers was not increased in the DES exposed cohorts [57]. Slight increases in the incidence of hypospadias and cryptorchidism have also been noted in DES exposed males, although the absolute risk is considered to be small [58, 59]. DES is unusual in being an oestrogen and also a mutagen. The influence of DES exposure *in utero* on breast cancer incidence has also been considered. A large study did not find an association between DES exposure and breast cancer in women under 40 years of age, but the finding of a non-statistical excess risk in a sub-set of women was considered to merit further work and for exposed women to enrol in cervical and breast screening programs [60].

Precursor or Substrate Depletion

The provision of nutrients at appropriate levels to the developing foetus by the mother is crucial to development. An example of nutritional status impacting development is maternal hypothyroidism as a result of iodine deficiency ([61, 62]). The human foetus derives thyroid hormones initially from the mother before attempting to synthesis its own from around 10-12 weeks [63]. These thyroid hormones are essential for pre- and postnatal brain development, and so severe maternal deficiencies may lead to mental impairment in the child, with congenital cretinism occurring in the most pronounced cases [64].

Folate Metabolism

Tetrahydrofolate is required in one–carbon transfer reactions in the synthesis of nucleic and amino acids and is produced by the reduction of folate by dihydrofolate reductase (DHFR) [41]. Consequently, blockade of folate supply at any stage could perturb RNA and DNA synthesis. This pathway has been exploited in the treatment of neoplasia, using DHFR inhibitors such as methotrexate (MTX, a methyl aminopterin derivative). MTX is also teratogenic and so this mechanism is plausible [39]. Anti-epileptic drugs (AED's) such as sodium valproate may cause neural tube defects and several interactions between AED's and folate have been proposed [65].

A key multi centre study conducted in the 1991 in the UK by the UK MRC Vitamin Study Research Group found that maternal periconceptional supplementation in 1195 high risk pregnancies with folic acid reduced the re-occurrence risk of neural tube defects (NTDs) significantly, suggesting 72% of NTDs were prevented [66]. From this study and expert advice thereafter, the UK Food Standards Agency's (FSA) recommend that: "if you are pregnant or thinking of having a baby you should take a daily 0.4 mg (400 microgram) folic acid supplement from the time you stop using contraception until the 12th week of pregnancy"[67]. A reduction in the occurrence of neural tube defects (NTDs) in all pregnancies by up to two-thirds by improving folate status has been suggested recently [68, 69].

The fact that not all NTDs are prevented even with peri-conceptual supplementation suggests genetic involvement in certain cases [70, 71]. Identifying these genetic factors in the population would allow for highly directed interventions in cases where NTDs would be resistant to folate [70].

The linking mechanistic factors between interference with folate and clinical outcomes is not entirely understood [41]. However, there is significant and developing evidence to indicate its importance in development and, issues concerning maternal folate supplementation aside, folate pathways may be one particularly important area for further work to be conducted.

Morphogenic Pathways

Foetal development requires the interaction and integration of a wide number of pathways for cell proliferation, cell differentiation and apoptosis to ensure the appropriate morphology of the neonate. Retinoid and Sonic Hedgehog (Shh) are just two signalling pathways that have been associated with teratogenic outcomes.

Retinoid

Reviews on the complex role of retinoids in development have been published [72, 73].

Retinoic acid (RA) is of importance as a morphogen in normal development and activates gene transcription throughout the embryo via a number of specific receptors [72]. Furthermore, different genes may be activated by different concentrations of retinoic acid and so its distribution and concentration are under precise local control [72]. The precursor molecule, retinol (Vitamin A) is converted to RA by retinol dehydrogenases and then retinal dehydrogenases [74]. The resulting RA then enter the nucleus and binds specific nuclear receptors; Retinoid X Receptor (RXR) and Retinoic Acid Receptor (RAR) which may then activate gene transcription via an autocrine pathway [74, 75]. One such family of retinoid–responsive genes is the Homeobox gene family, which are crucial in cellular differentiation [72, 76].

Exogenous retinoids such as Vitamin A (retinol), at relatively high doses, and a number of retinoic acids are well known teratogens and either bind these receptor sites or function as precursors for metabolites that are able to bind these sites [75]. The interaction of synthetic retinoic acid or their metabolites with the retinoic acid receptor (RAR) or at receptors not yet identified [77] modifies gene expression by affecting transcriptional activation and gene product production [74]. Neural crest cells are particularly affected, with changes in morphogenetic specification resulting in cranio-facial abnormalities [78]. Treatment of mice with RA can also result in a high incidence of spina bifida, which may arise from excessive apoptosis of the posterior neural plate neural crest cells where only limited programmed cell death should normally occur [73].

An example of a potent retinoid affecting humans is 13-*cis*-Retinoic acid (Isotretinoin or Accutane™), which has been used in treating cystic acne [79] and occasionally causes abnormalities in spite of recommendations for effective contraception before treatment and discontinuation during pregnancy [63]. A number of case studies detailing outcomes after isotretinoin exposures have been reported [80, 81].

Sonic Hedgehog (Shh) Protein

The secreted protein, Shh, is essential for multiple events in embryogenesis, including correct craniofacial development, with mutations in both mice and humans causing distinctive facial and brain abnormalities [82]. Defects in the Shh pathway has also been implicated in the formation of oesophageal atresia, and that this defect may possibly be attributable to environmental rather that genetic causes [83]. This pathway also offers some insight into the mechanism of ethanol teratogenicity: the neural crest cell death seen in chick embryos with Shh signals blocked by antibodies is similar to

that seen in a number of species after ethanol exposure *in-vitro* and *in-vivo* [82]. The expression of Shh may also be increased in the developing chick limb-bud by retinoic acid [73]. These and other data support the roles of Shh in normal craniofacial development [84].

Intra-Uterine Growth Restriction (IUGR): Disruption of Growth Hormone- Insulin – Like Growth Factor (GH-IGF) Axis

Intrauterine growth retardation has been associated with a number of perinatal complications and morbidity in later life including glucose tolerance and diabetes [85]. The GH-IGF axis is of primary importance in the foeto-placental growth and development and levels of IGF may be detected from the first trimester, with levels increasing throughout pregnancy [86]. Studies in transgenic animals null for genes encoding IGFs have revealed significantly decreased body weights in the offspring when compared with wild type animals [87]. The concentration of foetal IGF *in-vivo* has also been positively correlated with birth weight in a number of species including humans [88].

This pathway will need further consideration should IUGR become an area of interest for work in the future. Additionally, interference with this pathway has also been implicated in the mechanism of action of thalidomide, whereby angiogenesis is perturbed leading to the classic signs of phocomelia (abbreviated limb development) [40]. The anti-angiogenic and other properties, such its anti-proliferative characteristics have, however, seen renewed and increasing clinical applications for thalidomide [89].

Sex Hormone Pathways

There has been concern regarding the effects of environmental chemicals on sexual development due to effects on sex hormone levels. Such chemicals are often referred to as endocrine disrupting chemicals (EDC). Much of the concern derives from wildlife and animal studies, where gonadal abnormalities etc. have been noted either in association with an exposure or following administration of particular chemicals. The key issue is whether many of the *in-vivo* or *in-vitro* changes noted in experimental species are in fact toxicological endpoints relevant to humans. That is to say, that the disruption has an adverse health outcome for the neonate or population. The key text on the subject of EDCs remains the WHO International Programme on Chemical Safety (IPCS) Global Assessment of Endocrine Disruptors [90], which while accepting the biological plausibility of risks to human health, did not consider there to be firm evidence of direct causal associations between low level exposure (i.e. levels measured in the general population) and adverse health outcomes [90].

The androgen and oestrogen receptors are typical members of the superfamily of receptors that includes steroid/thyroid/retinoid/D_3 (zinc finger) receptors [41]. As such, ligand binding by the nuclear receptor results in activation of specific hormonal response elements result in increased transcription of appropriate genes. Outcomes therefore from either potent steroid agonist or antagonist ligands may be anticipated. However, there are a number of other mechanisms through which sexual development may be affected; as outlined below.

Testosterone Biosynthesis

Administration of certain phthalate compounds to experimental animals may cause abnormalities such as underdeveloped nipples, hypospadias and cryptorchidism and also testicular damage [91].

In the rat, transcription of genes involved in both cholesterol transport (testosterone precursor) and the biosynthesis of testosterone is affected by di-butyl phthalate treatment [92]. It is presently unknown whether our current exposure levels to certain phthalate (which may affect testosterone biosynthesis in animal models) have any consequences for human reproductive health [93].

Steroid Hormone Metabolism: Sulphotransferase (SULT) Effects

The levels of active steroid hormones is regulated in part by the enzymatic action of sulfotransferases, which conjugate a sulphonate (SO_3^-) group with a steroid to inactivate it [93]. A number of environmental chemical compounds have been have been shown to inhibit this process at relatively high dose levels including alkyl phenols [94], and a number of polyaromatic hydrocarbons [95]; which in turn leads to a relative increase in steroid levels by prolonging the effective half-life in tissues [93]. Whether these changes are of significance in human development is, however, not clear.

Oxidative Damage: Macromolecular Damage by Generation of Reactive Oxygen Species (ROS)

Reactive oxygen species are a feature of normal human metabolism, and protective and repair mechanisms necessarily exist to counter their effects. ROS have been implicated in an ever increasing number of disease states such as atherosclerosis, stroke and cancer. Wells et al 2005 provide a broad review of a number of pathways that involve reactive oxidative species in teratogenesis [96]. The rapid proliferation of cells during embryonic development, together with an underdeveloped metabolic complement may make the embryo particularly susceptible to oxidative stress. ROS have been implicated in the mechanisms of a number of teratogens including ethanol [97], phenytoin [98], and thalidomide [99]. However, ROS are only part of the mechanism of these teratogens, and the specific targets are not well defined.

Maternal Physiological Changes

These types of effects are perhaps most relevant when considering overt maternal effects by potent ligands affecting maternal physiology. Examples here may include the effects of drugs of abuse such as cocaine or alcohol on placental blood flow and other pressor effects and as such will not be discussed further.

Conclusion – Chemical Teratogenesis

The causal factor in the majority of developmental abnormalities is not known. This in itself makes delineation of chemically influenced birth outcomes complex. Where teratogens have been identified from animal experimentation, there is limited understanding of the mechanism of teratogenesis which is particularly the case for non-pharmaceutical chemicals. Mammalian development is highly complex and involves a multitude of gene expressions and metabolic pathways. Changes to any number of processes, especially at an early stage of development, may potentially have profound outcomes.

Section 3: Chemicals Implicated as Reproductive Toxicants; Consideration of the Evidence of Environmental Exposures

A number of chemicals have been classified already by national and international authorities for their possible impacts on the unborn child. Where classification occurs within the EU (see Table 6), corresponding risk management measures are taken. For instance, a given chemical classified as Category 1 or 2 on the basis of its toxicity to the reproductive system will be prohibited from use in consumer products used by the general public. While the emphasis of this review has been on teratogenesis, EU listings for chemicals that that could affect fertility have been included for completeness. These authoritative listings of classified chemicals may also be a useful reference for the Agency in detailing chemicals that may be of special toxicological significance in chemical incidents, for example.

Concerns have been expressed regarding the impact of chemicals in the environment on public health. Specific concerns include the potential health effects from drinking water disinfection bi-products and land fill waste sites. Consideration of the evidence regarding environmental exposure from these is also presented in this section.

Classification of Reproductive Toxicants

The system by which chemicals are classified in the EU is one based on hazard, and not risk. That is to say that is based on inherent properties of the chemical, and it does not take into account exposure in use, and hence risk.

This hazard-based classification system used by all EU Member Countries is defined in Annex VI to Commission Directive 67/548/EEC (Anon 1967) [100] and amendments. The chemical lists were derived from harmonised classifications and labelling for substances or groups of substances derived from Annex I to Directive 67/548/EEC on Classification and Labelling of Dangerous Substances. These classifications are legally binding within the EU and are regularly updated, with revisions arising from advice of the Technical Committee for Classification and Labelling (TC C&L), with participation of experts from Member States. Their meetings are prepared, chaired and followed-up by the European Chemical Bureau (ECB), which provided the online database for these data [101]. The data considered in the following paragraphs was derived on 10 August 2005, and the ECB source data will require periodic review. This Directive is implemented in the UK by the Health and Safety Executive (HSE) and the CHIP Regulations (Chemicals [Hazard Information and Packaging for Supply], Regulations 2002).

In the EU system, reproductive toxicity is divided into effects on fertility and developmental toxicity. In addition, substances which may interfere with lactation or be present in the breast milk in significant amounts can be classified as hazardous to breast-fed neonates. There are three separate categories used, which are defined in Table 6.

Table 6: Categories of Reproductive Toxicant as defined in Annex I to Directive 67/548/EEC on Classification and Labelling of Dangerous Substances

Category of Reproductive Toxicant	Basis of Classification
Category 1	
(i) Substances known to impair fertility in humans	Sufficient evidence to establish a **causal relationship** between human exposure to the substance and impaired fertility
(ii) Substances known to cause developmental toxicity in humans	Sufficient evidence to establish a **causal relationship** between human exposure to the substance and subsequent developmental toxic effects in the progeny.
Category 2	Sufficient evidence to provide a **strong presumption** that human exposure to the substance may result in:
(i) Substances which should be regarded as if they impair fertility in humans	(a), Impaired fertility on the basis of: clear evidence in animal studies of impaired fertility in the absence of toxic effects, or, evidence of impaired fertility occurring at around the same dose levels as other toxic effects but which is not a secondary non-specific consequence of the other toxic effects; other relevant information
(ii) Substances which should be regarded as if they cause developmental toxicity in humans	(b), Developmental toxicity, generally on the basis of: clear results in appropriate animal studies where effects have been observed in the absence of signs of marked maternal toxicity, or at around the same dose levels as other toxic effects but which are not a secondary non-specific consequence of the other toxic effects; other relevant information.
Category 3	Results in appropriate animal studies which provide sufficient evidence to cause a **strong suspicion** of:
(i) Substances which cause concern for human fertility	(a), Impaired fertility in the absence of toxic effects, or evidence of impaired fertility occurring at around the same dose levels as other toxic effects, but which is not a secondary non-specific consequence of the other toxic effects, but where the evidence is insufficient to place the substance in category 2, and/or other relevant information
(ii) Substances which cause concern for humans owing to possible developmental toxic effects	(b), Developmental toxicity in the absence of marked maternal toxicity, or at around the same dose levels as other toxic effects but which are not a secondary non-specific consequence of the other toxic effects, but where the evidence is insufficient to place the substance in category and/or other relevant information.

The chemical listings below (Tables 7 and 8) are derived from the EC Category 1 or 2 chemicals toxic to reproduction only, as these incorporate the chemicals for which there is most concern.

Table 7: Category 1 and 2 Reproductive Toxicants, Based on Fertility Data and Assigned Risk Phrase R60: May Impair Fertility

Substance Name
potassium dichromate
ammonium dichromate
sodium dichromate anhydrate
sodium dichromate, dihydrate
sodium chromate
cadmium fluoride
cadmium chloride
cadmium sulphate
benzo[a]pyrene, benzo[def]chrysene
1-bromopropane, n-propyl bromide
1,2-dibromo-3-chloropropane
1,2,3-trichloropropane
2-bromopropane
2-methoxyethanol, ethylene glycol monomethyl ether
2-ethoxyethanol, ethylene glycol monoethyl ether
1,2-dimethoxyethane, EGDME, ethylene glycol dimethyl ether
2,3-epoxypropan-1-ol, glycidol, oxiranemethanol
bis(2-methoxyethyl) ether
R-2,3-epoxy-1-propanol
4,4-isobutylethylidenediphenol
2-methoxyethyl acetate, methylglycol acetate
2-ethoxyethyl acetate, ethylglycol acetate
N-3,5-dichlorophenyl-5-methyl-5-vinyl-1,3-oxazolidine-2,4-dione, vinclozolin (ISO)
methoxyacetic acid
DEHP, bis(2-ethylhexyl) phthalate, di-(2-ethylhexyl) phthalate
1,2-benzenedicarboxylic acid, dipentylester, branched and linear [1]: n-pentyl-isopentylphthalate [2]: di-n-pentyl phthalate [3]: diisopentylphthalate [4]
carbendazim (ISO), methyl benzimidazol-2-ylcarbamate
benomyl (ISO), methyl 1-(butylcarbamoyl)benzimidazol-2-ylcarbamate
3-ethyl-2-methyl-2-(3-methylbutyl)-1,3-oxazolidine

These chemicals are as defined in Annex I to Directive 67/548/EEC on Classification and Labelling of Dangerous Substances (as at August 2006).

Table 8: Category 1 and 2 Reproductive Toxicants, Based on Developmental Toxicity Data and Assigned Risk Phrase R61: May Cause Harm to the Unborn Child

Substance Name
carbon monoxide
3-(3,4-dichlorophenyl)-1-methoxy-1-methylurea, linuron (ISO)
lead hexafluorosilicate
6-(2-chloroethyl)-6-(2-methoxyethoxy)-2,5,7,10-tetraoxa-6-silaundecane, etacelasil
bis(4-fluorophenyl)(methyl)(1H-1,2,4-triazol-1-ylmethyl)silane, flusilazole (ISO)
A mixture of: 4-[[bis-(4-fluorophenyl)methylsilyl]methyl]-4H-1,2,4-triazole; 1-[[bis-(4-fluorophenyl)methylsilyl]methyl]-1H-1,2,4-triazole
potassium dichromate
ammonium dichromate
sodium dichromate anhydrate
sodium dichromate, dihydrate
sodium chromate
nickel tetracarbonyl, tetracarbonylnickel
cadmium fluoride
cadmium chloride
cadmium sulphate
lead compounds with the exception of those specified elsewhere in this Annex
lead alkyls
lead azide, lead diazide
lead chromate
lead di(acetate)
trilead bis(orthophosphate)
lead acetate, lead acetate, basic
lead(II) methanesulphonate
C.I. Pigment Yellow 34, Lead sulfochromate yellow, [This substance is identified in the Colour Index by Colour Index Constitution Number, C.I. 77603.]
C.I. Pigment Red 104, Lead chromate molybdate sulfate red, [This substance is identified in the Colour Index by Colour Index Constitution Number, C.I. 77605.]
lead hydrogen arsenate
benzo[a]pyrene, benzo[def]chrysene
diphenylether; octabromo derivate
2-methoxyethanol, ethylene glycol monomethyl ether
2-ethoxyethanol, ethylene glycol monoethyl ether
1,2-dimethoxyethane, EGDME, ethylene glycol dimethyl ether
2-methoxypropanol
bis(2-methoxyethyl) ether
1,2-bis(2-methoxyethoxy)ethane, TEGDME, triethylene glycol dimethyl ether, triglyme
tetrahydrothiopyran-3-carboxaldehyde
2-methoxyethyl acetate, methylglycol acetate
2-ethoxyethyl acetate, ethylglycol acetate
warfarin [1]: (S)-4-hydroxy-3-(3-oxo-1-phenylbutyl)-2-benzopyrone [2]: (R)-4-hydroxy-3-(3-oxo-1-phenylbutyl)-2-benzopyrone [3]
2-ethylhexyl[[[3,5-bis(1,1-dimethylethyl)-4-hydroxyphenyl]methyl]thio]acetate
bis(2-methoxyethyl) phthalate
2-methoxypropyl acetate

These chemicals are as defined in Annex I to Directive 67/548/EEC on Classification and Labelling of Dangerous Substances (as at August 2006).

Table 8 (continued): Category 1 and 2 Reproductive Toxicants, Based on Developmental Toxicity Data and Assigned Risk Phrase R61: May Cause Harm to the Unborn Child

Substance Name
butyl (RS)-2-[4-(5-trifluoromethyl-2-pyridyloxy)phenoxy]propionate, fluazifop-butyl (ISO)
N-3,5-*dichlorophenyl*-5-methyl-5-vinyl-1,3-oxazolidine-2,4-dione, vinclozolin (ISO)
methoxyacetic acid
DEHP, bis(2-ethylhexyl) phthalate, di-(2-ethylhexyl) phthalate
DBP, dibutyl phthalate
(±) tetrahydrofurfuryl (R)-2-[4-(6-chloroquinoxalin-2-yloxy)phenyloxy]propionate
1,2-benzenedicarboxylic acid, dipentylester, branched and linear [1:] n-pentyl-isopentylphthalate [2]: di-n-pentyl phthalate [3]: diisopentylphthalate [4]
BBP, benzyl butyl phthalate
1,2-benzenedicarboxylic acid, di-C7-11-branched and linear alkylesters
A mixture of: disodium 4-(3-ethoxycarbonyl-4-(5-(3-ethoxycarbonyl-5-hydroxy-1-(4-sulfonatophenyl)pyrazol-4-yl)penta-2,4-dienylidene)-4,5-dihydro-5-oxopyrazol-1-yl)benzenesulfonate, trisodium 4-(3-ethoxycarbonyl-4-(5-(3-ethoxycarbonyl-5-oxido-1-(4-sulfonatophenyl)pyrazol-4-yl)penta-2,4-dienylidene)-4,5-dihydro-5-oxopyrazol-1-yl)benzenesulfonate
lead 2,4,6-trinitro-m-phenylene dioxide, lead 2,4,6-trinitroresorcinoxide, lead styphnate
dinocap (ISO)
2-sec-butyl-4,6-dinitrophenyl-3-methylcrotonate, binapacryl (ISO)
6-sec-butyl-2,4-dinitrophenol, dinoseb
salts and esters of dinoseb, with the exception of those specified elsewhere in this Annex
2-tert-butyl-4,6-dinitrophenol, dinoterb (ISO)
salts and esters of dinoterb
2,4-dichlorophenyl 4-nitrophenyl ether, nitrofen (ISO)
methyl azoxy methyl acetate, methyl-ONN-azoxymethyl acetate
2-[2-hydroxy-3-(2-chlorophenyl)carbamoyl-1-naphthylazo]-7-[2-hydroxy-3-(3-methylphenyl)carbamoyl-1-naphthylazo]fluoren-9-one
azafenidin
2,6-dimethyl-4-tridecylmorpholine, tridemorph (ISO)
2-imidazoline-2-thiol, ethylene thiourea, imidazolidine-2-thione
carbendazim (ISO), methyl benzimidazol-2-ylcarbamate
benomyl (ISO), methyl 1-(butylcarbamoyl)benzimidazol-2-ylcarbamate
cycloheximide
N-(7-fluoro-3,4-dihydro-3-oxo-4-prop-2-ynyl-2H-1,4-benzoxazin-6-yl)cyclohex-1-ene-1,2-dicarboxamide , flumioxazin (ISO)
A mixture of: 1,3,5-tris(3-aminomethylphenyl)-1,3,5-(1H,3H,5H)-triazine-2,4,6-trione, a mixture of oligomers of 3,5-bis(3-aminomethylphenyl)-1-poly[3,5-bis(3-aminomethylphenyl)-2,4,6-trioxo-1,3,5-(1H,3H,5H)-triazin-1-yl]-1,3,5-(1H,3H,5H)-triazine-2,4,6-trione
N,N-dimethylformamide, dimethyl formamide
N,N-dimethylacetamide
formamide
N-methylacetamide
N-methylformamide

These chemicals are as defined in Annex I to Directive 67/548/EEC on Classification and Labelling of Dangerous Substances (as at August 2006).

Consideration of Evidence Regarding Environmental Exposure

There are a number of concerns with regards to environmental exposures to chemicals and possible effects on reproductive health. These arise in 2 main areas, namely from drinking water disinfection bi-products and landfill waste sites. These are considered below.

Disinfection by-products

There is currently a debate on the potential toxicity of water containing chlorinated disinfection by-products (DBPs) [102]. Chlorination is used around the world as an effective microbial disinfection agent for drinking water. Trace organic matter (i.e. plant and vegetable matter in surface water may, however, react with chlorine during treatment to form a number of organic halogens; primarily trihalomethanes (THM) and also haloacetic acids (HAA) and halonitriles [102]. In 1998 the Committee on the Toxicity Chemicals in Food, Consumer Products and the Environment (COT) considered findings of epidemiology studies conducted in California which reported an association between high consumption of tap water and the incidence of spontaneous abortion [103]. COT considered there was insufficient evidence but recommended further work. A statement by the COT in 2004 concluded that there was no causal relationship in the data evaluated, and recommended work to reduce uncertainties between patterns of intake and the incidence of reproductive outcomes (COT/04/8 November 2004).

The COT recommended the following:

"We *recommend*, however, further research to reduce uncertainties in the interpretation of the reported associations between patterns of drinking-water intake and the incidence of adverse reproductive outcomes. In particular, we *recommend* prospective study designs which include more precise assessment of individual exposures, allowance for seasonal variations in chlorination byproduct concentrations, and more comprehensive analyses of the influence of other potential causative agents and confounding factors."

The Small Area Health Statistics Unit (SASHU) at Imperial College, London is currently conducting work on this area including consideration of congenital abnormalities, and the COT will further review the area when the reports of these studies are available.

A summary of the COT view on chlorinated tap water and the unborn child is as follows:

"Disinfection of tap water by chlorination is an important measure to protect public health. Government recognises concerns that chlorinated tap water may harm the unborn child, and is guided by the advice of its independent scientific advisors. Following a further review of the scientific information, including results of Government-funded research, the Committee on Toxicity concluded that the data do not show that chlorinated drinking-water affects pregnancy outcomes. The Committee suggested further lines of research, and again endorsed precautionary measures by water companies to minimise consumers' exposure to chlorination byproducts in tap water, providing that they do not compromise the efficiency of disinfection of drinking-water."

Landfill Waste Sites

Despite the legislative controls on landfill sites, concern continues to be expressed about whether they might present a health risk for people living nearby. A number of scientific studies have investigated whether there are higher than usual incidences of adverse health events, such as cancer or congenital anomalies (birth defects), in populations living near to sites. To date, no clear picture has emerged. Many of these studies investigated old sites, uncontrolled dumps or sites where significant off-site migration of chemicals was detected, and the results do not necessarily apply to landfill sites in general or, in particular, to landfill sites meeting present-day standards.

In August 1998 a study of the incidence of congenital anomalies near "hazardous waste" landfill sites in Europe (the EUROHAZCON study) was published in the Lancet [104]. This study investigated pregnancy outcomes in women living within 7 kilometres of 21 hazardous waste landfill sites in five countries, including the United Kingdom. Overall, it found an increased incidence of non-chromosomal congenital anomaly i.e. birth defects not caused by changes in the structure of chromosomes, which carry the genetic material and specifically neural tube defects and abnormalities of the great arteries and veins, in neonates whose mothers lived close to a landfill site, compared to neonates of those mothers who lived further away. The authors concluded that there was a need for further investigation to determine whether this meant that landfill sites contribute to the risk of these birth defects i.e. that the landfill sites were causal in the birth outcomes.

In response to the concerns raised, Government Departments commissioned SAHSU to carry out a large study of birth outcomes and cancer around landfill sites in Great Britain. The study found a small increase in congenital anomalies in populations living close to landfill sites, but the increase (1% higher than the reference population for all sites but 7% around special waste sites)) was much smaller than had been reported in the EUROHAZCON studies [105]. With respect to specific abnormalities an increased risk ratio was seen for neural tube defects, hypospadias/epispadias and abdominal wall defects. An increased risk of low birth weight was also seen in the study population. There was no suggestion of excess risks of cancer associated with landfill sites. The COT also considered this study and noted that the findings for birth outcomes were not consistent, and that the study provided no evidence that the rates of anomalies increased after sites had opened. A detailed COT statement on the study can be found online [106].

In addition, results from subsequent EUROHAZCON studies have been reported. In January 2002, Vrijheid *et al* reported an increased incidence of birth defects due to chromosomal congenital anomalies (a category which includes Down's syndrome) in neonates whose mothers lived close to a 'hazardous waste' landfill site [107]. However this study was of the same design as the first study and therefore subject to the same difficulties in interpretation as noted above. In November 2002, a further study by the same group found "little evidence" for a relationship between risk of congenital anomaly and a rough measure of the "hazard potential" of landfill sites as judged by an expert panel [108]. A further SASHU study reported in 2003 considered populations resident within 2 km of 61 special landfill sites in Scotland and concluded that there was no statistically significant excess risk of congenital abnormalities or low birth weight [109].

Government Research Relating to Landfill Sites and Health Effects

In January 1999 the Department of Health and the Department of the Environment, Transport and the Regions sponsored a meeting of invited experts at the Medical Research Council Institute of Environment and Health to discuss the data on landfill sites and health effects, to identify gaps in knowledge, and to agree priorities for further research to fill these gaps. Subsequently, the Government announced a new research programme on the impact of landfill sites on health. This was designed to provide further scientific data to support the development of Government policy on landfill in general and to inform the debate on the possible effects on human health of landfill. Details of this research programmed are available online [110].

Part of this work involved a comprehensive review of potential developmental toxicity of chemicals possible released from landfill sites and was published in 2001. A number of chemicals were considered in terms of their experimental data, human case reports and epidemiology [111]. The research programme also includes a comprehensive review of the known causes of congenital anomalies of concern, in particular those relating to the environment to help put into context the results on congenital anomalies and landfill sites, again this work is now available [3].

Also available is a study examining whether geographic variation exists in the overall rates of congenital abnormalities and in the rates of specific congenital abnormalities. This study of 5 regions of Britain, over the period 1991-99 and covering 840,000 births, found little evidence of clustering of congenital abnormalities [112].

In addition, there is a large project being carried on behalf of the Environment Agency, on exposure assessment. Detailed monitoring of emissions from landfill sites is being carried out in order to assess the exposure of people living and working nearby. The environmental work study is complete and data analysis and interpretation is underway, but some difficulties have arisen. It is not known at this stage when a final report with conclusions will be available.

It is anticipated that the results of the results of the environmental monitoring study, and the information in the reviews will contribute information that may lead to further more focused studies in this area to enable better understanding of this issue.

Section 4: Detection of Effects on Fertility after Chemical Exposures

Situations arise due to accidents or other causes where individuals may be acutely exposed to chemicals that could potentially affect reproductive health. In response to this type of incident in the UK, expertise may be sought from the National Teratology Information Service (NTIS) based in Newcastle.

It is pertinent to note in this section that a number of studies have reported a temporal decline in human sperm quality in several countries, but the evidence is inconsistent and the issue remains controversial [90].

It is possible to determine exposure and uptake by biomonitoring studies using a variety of body fluids, e.g. benzene metabolites in semen [113]. Linking exposures to infertility is complicated even after documented acute exposures to chemicals that may cause harm, as approximately 14-17% of couples encounter fertility problems during their reproductive years [1, 2]. Of this incidence, 40% are possibly attributable to male factors, and many of these causes are unknown and are considered as idiopathic [114]. Fertility measures after a chemical incident by success in conceiving would not be available for up to a year, or the statistical power of such an investigation would be low due to the small numbers of people likely to be involved in most incidents [115]. These studies following-up on an incident are useful for future scenarios or for determining long term health issues, but are not appropriate for advising the individuals exposed at that time. From a population perspective, it would be ideal if early indicators could be detected that precede measurable changes in fertility [116] and it is desirable to predict fertility or damage to sperm at an early stage.

Biomarkers that could assist in determining the impacts of chemical exposure on fertility would be a useful tool in response to certain chemical incidents. With this knowledge, more appropriate and accurate and indeed rapid advice may be provided to individuals. The rationale for focusing on male fertility in this section of the review is based to large extent on the greater information in this area due to the relative ease of studying effects on the male gametes. In addition, there are specific concerns regarding the continuous nature of gamete production and the associated vulnerability. The male gametes are also readily accessible for assessment following a putative exposure in the workplace, for example when compared with female gametes. This accessibility also assists with prospective work; although it should be noted that recruitment into studies that require volunteers to provide semen samples is problematic for a number of reasons. It would be desirable for any biomarker of effect to provide information on the extent of any recovery from damage as well as the nature of initial damage; though this would clearly require appropriate clinical counselling support.

The following text will consider possible strategies for determining the effects of exposure to chemicals of concern on male fertility. The majority of the work in this area has not, however, been conducted with regards to chemically exposed populations. The standard assessments made of semen quality in current practice will not be considered in detail; reference should be made to relevant WHO assessment criteria [117].

Infertility and Sperm Damage Assessment

There are many targets for toxicity that may affect fertility; a selection of physiological biomarkers that may have the most relevance for infertility are presented (Table 9).

Table 9: Biomarkers of Physiologic Reproductive Toxicity in Men Related to Effects on Male Fertility, Adapted from Wyrobek, 1997 [115]

Type of Tissue, Sample or Source	Type of Measurement
Blood	Hormone Levels
Sperm	Concentration and total count Morphology and computer determined morphometry Motility (visual and Computer Assisted Sperm Analysis [CASA]) Viability Agglutination Penetration and Interaction: Cervical Mucus Hamster Eggs Non-living human eggs Internal and surface domains Chromatin Structure Biochemical Measurements
Semen	Physical Characteristics Chemical Composition (normal and xenobiotics substance) Immature germ cells Cellular function measures Sertoli cells Leydig cells Accessory glands
Testes	Histopathology
Survey and medical records	Standardized fertility ratio

Assessment of fertility has traditionally focused on semen-quality and early sampling of semen may be particular useful in assessing the induction of damage in mature sperm in the vas deferens and epididymis or if accessory sex glands are affected [115]. Biomarkers have been identified in blood for possible use. Semen is clearly ideal for use in analysis, as it is readily accessible. Sperm or seminal content may be associated with changes in fertility potential that may be relevant to the individual and defects in sperm DNA or chromosomes may be associated with detrimental effects on the viability of the embryo and subsequent health risks to the newborn [115]. Semen and the sperm therein can be assessed for a number of parameters (Table 9) and provides a range of information including:

- Sperm production (numbers of sperm, normal morphology)
- Sperm function (viability, vigour and normal morphology)
- Specific semen endpoints
- Sensitive and specific biomarkers [115] (See below)

In humans, sperm take approximately 75 days to develop from a stem cell into a sperm within the seminiferous vesicles; and a further 12 days to travel through the epididymides to the vas deferens [115]. Accurate assessments of outcomes following toxicity must therefore consider this lag phase of almost 90 days and use serial sperm sampling as a means of determining gross insult, and thereafter possible recovery.

On exposure to a chemical toxicant, sperm will be at different stages of development and are likely to have different susceptibilities. For example, as rapidly dividing cells, the spermatogonia are particularly susceptible, followed by the spermatocytes [118]. Toxicity at the stem cell level will, necessarily have the most pronounced effect on spermatogenesis, though other exposures may be significant.

The use of Computer Assisted Sperm Analysis (CASA) should prove useful in conducting standard assessments of sperm, and may allow for more rapid and consistent evaluations when considering large numbers of samples [119].

Standard semen parameters, whilst a useful (gross) indicator of testicular or germ cell toxicity do not provide a complete assessment. For instance, the molecular functionality of the gametic DNA may be affected but not the motive performance of the sperm [120]. Furthermore, there is considerable individual variation in seminal parameters [121]. Clearly, additional methods of assessment are required to assist clinical decisions.

Specific Biomarkers for Toxicity to the Male Reproductive System

Characteristics of an ideal biomarker for measuring toxicity to a male reproductive system have been proposed [115]:

- A chemical, biochemical, or cellular factor that is a constituent of spermatozoa or semen (or other body fluid) that can be characterized objectively.

- The biological mechanism represented should be sufficiently well understood so that changes in the assay measurement can be directly related to changes in the function of cell or tissue being monitored (e.g., germ cell, Sertoli cell, Leydig cell, accessory glands, epididymides, efferent ducts).

- Ideally, it should also be understood how changes in the biomarker measurement are related to changes in fertility status.

With few exceptions, no biomarker has yet met all these criteria, but several approaches are under consideration and a number of possibilities are outlined in the following sections. It should be noted that recruitment into studies that require volunteers to provide semen samples is more problematic than for studies requiring assessments of urine or blood. The lack of control data as to what is “normal” is also limiting when starting to consider novel biomarkers. What is most likely is that several biomarkers may be required to identify the lesion that has occurred. Two areas of key interest are biochemical parameter alterations in the semen and sperm and changes to sperm DNA integrity.

Selected Biochemical Indicators of Toxicity

Urinary Creatine

This biochemical marker has been suggested as a non-invasive biomarker of testicular toxicity [122]. Animal studies have demonstrated that creatine may increase in the urine after exposure to testicular toxicants such as cadmium chloride and 2-methoxyethanol [122]. In rats, the creatine is released from the damaged testes into the blood steam and into the urine via the kidneys [123]. While this marker has some potential for assessing testicular toxicity, its relevance in man has not been considered widely or recently in the literature, and there are concerns about the lack of specificity.

P34H

A limited number of authors have considered the epididymal sperm protein P34H as a biomarker in fertility [124, 125]. This protein is acquired on the sperm surface on the acrosomal cap as they transit the epididymis , and is involved in the binding of the zona pellucida [114]. Though not defined in terms of chemical toxicity, this may serve as a possible biomarker of epididymal integrity.

Creatine Kinase (CK) and HspA2 Assays

The presence of CK in spermatozoa may be a useful biomarker of abnormal late development. Increased sperm CK reflects residual cytoplasm in spermatozoa that did not complete the developmental step of cytoplasmic extrusion [126]. Immature sperm populations with higher cytoplasmic content are more susceptible to oxidative damage by lipid peroxidation [115].

HspA2 (Formerly known as CK-M) is another protein expressed in spermatogenesis and associated with late stage development. Blinded clinical studies have revealed that a decrease in CK activity and decreased HspA2 were consistent with diminished male fertility [126]. Though not defined in terms of chemical toxicity, these parameters may possibly be sperm parameters that merit additional consideration.

Hyaluronic Acid

The ability to bind hyaluronic acid has been defined as a possible marker of sperm maturation and may be another useful indicator or competent sperm development and maturation in man [126].

Inhibin B

Determination of sex hormone levels is comparatively simple although interpretation of the significance of the results is difficult. A number of hormonal parameters have been considered, but these are poor correlates for spermatogenic potential e.g. Follicle stimulating hormone (FSH) is less appropriate for testicular assessment, as due to hypothalamic influences [127]. Individual hormone levels are subject to significant temporal changes and so ratios of different hormones may be more suitable [122].

Inhibin B has been proposed as a useful marker in spermatogenesis for use in population studies of male reproductive health [127] and may be of interest in a chemical incident context.

Inhibin B is secreted from Sertoli cells, and feeds back negatively to control pituitary secretion of FSH [128, 129]. The circulating concentration of inhibin B therefore quantitatively reflects Sertoli cell number and consequently sperm production [130]. Measurements of inhibin B levels have correlated well with impaired testicular function in infertility investigations, with the highest levels being found in fertile men [131]. Decreases in inhibin B levels have been demonstrated after exposure to radiotherapy or chemotherapeutic agents in both animal [132, 133] and in man [134-136].

There are differing opinions on the adequacy of inhibin B as a biomarker of spermatogenesis in infertile men [128, 137]; and some workers consider measurement of inhibin B in association with FSH to determining the type of damage most accurately [138]. However, inhibin B may be useful for the rapid identification of spermatogenic disorders in populations exposed to genetic toxicants [128].

Inhibin B concentration may be measured in both serum and in semen. A prospective study with chemotherapeutic treatment regimens in man has demonstrated decreased in inhibin B to 20% of baseline the interrelation of inhibin B levels with FSH, and the adequacy of blood sampling methodologies [139]. Much of the work conducted in this area relates to infertility clinics and not to chemical exposure and careful consideration may be required before a biomarker screen in developed.

Blood samples are easier to obtain than ejaculates for a larger population study. Where semen samples are practical, inhibin B concentrations may be a more reflective of the functional state of the seminiferous epithelium [140]. This approach may be more sensitive in determining the effects of lower levels of exposure or exposure to less potent agents.

DNA Integrity and Sperm Damage

Measurement of genetic damage is increasingly used in the search to understand abnormal reproductive function [116]. A significant body of the work conducted into DNA integrity and reproductive outcome stems from concerns raised from assisted reproductive technologies, notably, intra-cytoplasmic sperm injection (ICSI) [120], where sperm are largely selected on the basis of motility and gross morphology which could bypass physiologic selection processes [141] . A negative correlation between ejaculated spermatozoa has been demonstrated between DNA damage in ejaculated spermatozoa and success in fertilisation *in-vitro* or achieving a pregnancy using several measures of DNA integrity [142-144].

Biological strategies for coping with DNA damage in germ cells has been reviewed by Olsen et al (2005) [145] and a number of techniques are available to assess damage to nuclear DNA in mammals some of which are detailed below. For a review, refer to Fraser (2004) [142].

Fluorescence In-Situ Hybridisation (FISH)

This technique, applied to de-condensed sperm heads, is a widely used as an accurate technique to indirectly study the chromosomal constitution of spermatozoa [146, 147].

FISH analysis uses the hybridisation of chromosome-specific DNA probes labelled with fluorochromes to complementary DNA sequences on target chromosomes which are then detected under a fluorescence microscopy [148].

Single-cell Gel Electrophoresis or Comet Assay

This technique may detect oxidative DNA damage in a range of cell types and may be conducted under pH neutral conditions to identify double stranded breaks or under alkaline conditions to detect both double and single stranded breaks. Both strategies have been used widely to detect DNA damage in both humans and animals [142]. This technique is labour intensive and not well suited to large scale determinations of damage in sperm DNA. Preservation of fresh sperm samples in liquid nitrogen allows retention and subsequent analysis [149].

DNA-Adduct Detection

Work has been conducted to assess whether the presence DNA adducts are an early marker for sperm genotoxicity and infertility [150, 151]. In one study, ^{32}P post-labelling revealed an association between DNA adducts in sperm and male-factor infertility and while a functional association was not confirmed, the authors suggested that this may be a parameter for consideration in male-fertility [150].

Main Conclusions and Recommendations

The causal factor in the majority of developmental abnormalities is not known. This in itself makes delineation of chemically influenced birth outcomes complex. Where teratogens have been identified from animal experimentation, there is limited understanding of the mechanism of teratogenesis, particularly for non-pharmaceutical chemicals.

There are key data gaps in knowledge on the adverse effect of environmental chemicals, to which the public are exposed, relating to toxicity to the reproductive system and especially developmental toxicity. This is being addressed to some extent by the voluntary OECD initiative in this area, and will be addressed by the proposed EC Regulation REACH. It is important that the HPA is aware of the output of these programmes.

There is a need for ready access to robust health statistics data on congenital abnormalities across the UK so as to be able to investigate effect in local areas of interest. This is a key "building block" to effective analysis of health statistics in this area. With enhanced data bases, appropriate epidemiological work may be undertaken in the future.

In instances where there is evidence that the public is potentially exposed to a chemical of concern from environmental pathways, there will be a need to obtain exposure data to enable an appropriate risk assessment to be made. There may be a need to obtain biomonitoring data to determine information on uptake.

An understanding of normal embryological development and foetal development is important in this regard. CHaPD will identify and consider developing links with appropriate experts in this area.

Some consideration of underlying mechanisms of chemical teratogenesis is important and there may be the need for mechanistic studies to further understand the action of specific agents, and to refine risk assessments. The following generic pathways and interactions are felt to merit further consideration:

- Retinoid, Shh and associated pathways in morphogenesis
- Folate metabolism and outcomes of interference
- Non-sex hormone mediated endocrine changes

A number of teratogens are known to be involved in these pathways and are also of public concern. These areas for future work are of interest to other government organisations and opportunities to combine scientific resources, where appropriate, should be considered. CHaPD has established good links with FSA, HSE and EA, amongst others, which should assist in this where practicable.

There is considerable work underway in developing alternative testing strategies namely under the EU ReProTect Project. This should facilitate the screening of chemicals for reproductive toxicity to identify those of primary concern. It is likely that most progress will be made in pre-screening of early embryotoxicity through the use of combined strategies. For applications such as QSAR and DNA arrays, a sound understanding of mechanisms associated with teratogenicity is required before these technologies can be fully exploited within a regulatory context. The development and

validation of screening assays is outside the remit of the HPA, but CHaPD needs to be aware of progress in this area.

With regards to the potential effects on fertility, only limited data are available on mechanisms, and most published work relates to male fertility. This review has thus concentrated on this area. The mechanisms leading to biomarker changes are poorly understood at the biochemical or molecular level, even for changes in sperm counts. Sperm counts may be derived readily, though may not be particularly robust as an endpoint, as there is significant variability both within as well as between healthy men [116]. Sperm cells have the potential at least to provide a link between exposure-mediated damage to target organ tissues and changes in fertility. An example of this is determination of xenobiotics in semen and the counts of sperm in the sample. DNA damage assessments may be useful in assessing damage to sperm, and possibly any recovery. This should not be conducted in isolation, as the primary event is damage to the testes, and the wide range of conventional andrology investigations will be key in the assessment process. Inhibin B is a candidate for the assessment of injury after exposure to Sertoli cell toxicants. However, normal inhibin B or indeed FSH or other hormone measurements do not guarantee sperm quality. The use of multiple parameters is likely to be the most pertinent approach and in population studies may assist in identifying pre-existing pathologies.

While nutritional issues are not the prime remit of the HPA, the impact of this on the area of concern i.e. birth defects, necessitates comment. There is good evidence that appropriate folate supplementation may significantly reduce, though not eliminate, the risk of many neural tube defects. In this regard, whilst attempting to ascertain the impact of chemicals on many aspects of development, folate supplementation may be one area of public health protection where further emphasis could possibly be placed, and additional educational campaigns should be considered for women in the UK to affect a sustained awareness across the population. The recent announcements by the Food Standards Agency that fortification of flour with folic acid is under consideration are welcomed. Some research has suggested that recommendations for women to take supplements may not have sufficient profile or be sustained enough to achieve the potential reductions in incidences of neural tube defects [69, 152, 153]. Fortification, as a measure may, therefore, be a particularly useful in protecting the health of the child by enhancing folate status at the peri-conceptually stage; critical with so many pregnancies being unplanned. Furthermore, it would eliminate some of the socio-economic inequalities that is associated with dietary supplementation.

Equally ethanol, smoking and drugs of abuse negatively impact on development, necessitating a multi-agency or multi-disciplinary awareness. In the absence of further efforts to reduce risk factors in the population, it will be difficult (if not impossible) to establish where critical exposures may be occurring.

As a public health organisation, with a limited research capability; clinical relevance to current or daily public concerns must be balanced carefully with longer term scientific investigations. Where there is evidence of environmental exposure to teratogens, deriving exposure data in humans and an increased knowledge of developmental processes will assist the Agency in identifying vulnerable communities or individuals, in identifying and managing critical exposures and assist in assessing human health outcomes.

Recommendations for Research

Recommend adequate funding be made available to ensure the availability of robust data, to a consistent standard nationwide, on health statistics relating to congenital abnormalities. Such data are of key importance for epidemiological studies on the potential developmental toxicity of environmental chemicals.

Access to these robust health statistics on congenital abnormalities is a key requirement. It is recommended that steps be taken to ensure such data are available to support epidemiological studies and experimental research in this area.

In order to obtain as much information as possible from human data, the HPA should make full use of potential toxicity data on exposure to chemicals and pregnancy outcome available from the National Teratology Information Service (NTIS).

It is recommended that HPA support further research to increase knowledge of the effects of environmental chemicals on reproductive/developmental toxicity. It will be important when developing a research strategy in this area, to establish close links with centres of expertise in reproductive toxicology; collaborative research projects should be considered.

Key aspects of this could include:

- Building on current expertise relating to biomarkers to cover development of markers for exposure to and uptake of environmental chemicals of concern.
- Biomarkers of effect relating to testicular toxicity/male infertility.
- Generic pathways involved in human development and their association with teratogenesis.

Glossary of Institutions and Organisations

Committee on the Toxicity of Chemical in Food, Consumer Products and the Environment (COT)

The COT is an independent scientific committee that provides advice to Government Departments and Agencies on matters concerning the toxicity of chemicals. The sister committees of the COM and COC provide advice on the mutagenicity and carcinogenicity, respectively.
(Please see http://www.food.gov.uk/science/ouradvisors/toxicity/).

European Centre for the Validation of Alternative Methods (ECVAM)

ECVAM co-ordinates at the European level the independent evaluation of the relevance and reliability of tests for specific purposes, so that chemicals and products of various kinds, including medicines, vaccines, medical devices, cosmetics, household products and agricultural products, can be manufactured, transported and used more economically and more safely, whilst the current reliance on animal test procedures is progressively reduced.
(Please see http://ecvam.jrc.it/index.htm)

European Chemical Bureau (ECB)

The ECB provides scientific and technical support to the conception, development, implementation and monitoring of EU policies on dangerous chemicals. The ECB ensures the development of methodologies and software tools to support a systematic and harmonised assessment of chemicals addressed in a number of European directives and regulations.
(Please see http://ecb.jrc.it/)

European Concerted Action on Congenital Anomalies and Twins (EUROCAT)

EUROCAT is a European network of population-based registries for the epidemiologic surveillance of congenital anomalies and was started in 1979. More than 1.5 million births surveyed per year in Europe using 43 registries in 20 countries and covers 29% of European birth population. The registries are high quality multiple source registries, ascertaining terminations of pregnancy as well as births. EUROCAT is funded by the EC DG Health Public Health Programme.
(Please see http://www.eurocat.ulster.ac.uk/index.html)

Food Standards Agency (FSA)

The FSA is an independent Government department set up by an Act of Parliament in 2000 to protect the public's health and consumer interests in relation to food. The FSA provides advice and information to the public and Government on food safety from farm to fork, nutrition and diet. It also protects consumers through effective food enforcement and monitoring.
(Please see http://www.food.gov.uk/)

Health and Safety Executive (HSE)

The HSE is a government agency who's job is to help the Health and Safety Commission ensure that risks to people's health and safety from work activities are properly controlled. The Health and Safety Commission is responsible for health and safety regulation in Great Britain. The Health and Safety Executive and local government are the enforcing authorities of the legislation who work in support of the Commision.
(Please see http://www.hse.gov.uk/index.htm)

International Programme on Chemical Safety (IPCS)

The International Programme on Chemical Safety (IPCS), established in 1980, is a joint programme of three Cooperating Organizations - ILO, UNEP and WHO, implementing activities related to chemical safety. WHO is the Executing Agency of the IPCS, whose main roles are to establish the scientific basis for safe use of chemicals, and to strengthen national capabilities and capacities for chemical safety.
(Please see http://www.who.int/ipcs/en/)

National Teratology Information Service (NTIS)

NTIS is based in the Northern and Yorkshire Regional Drug and Therapeutics Centre in Newcastle upon Tyne. It is funded by the Health Protection Agency to provide a national, 24 hour service on all aspects of toxicity of drugs and chemicals in pregnancy to medical professionals.
(Please see http://www.ncl.ac.uk/pharmsc/entis.htm)

Organisation of Economic Co-operation and Development (OECD)

This us a forum where the governments of 30 market democracies work together to address the economic, social and governance challenges of globalisation as well as to exploit its opportunities. This includes the production of a range of internationally agreed instruments, decisions and recommendations.
(Please see http://www.oecd.org/home/0,2987,en_2649_201185_1_1_1_1_1,00.html)

Small Area Health Statistics Unit (SASHU)

The main aim of SAHSU has been to assess the risk to the health of the population to environmental factors with an emphasis on the use and interpretation of routine health statistics. This unit based within the Epidemiology and Public Health Department of Imperial College, London and is funded by Government.
(Please see http://www.sahsu.org/)

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