

CHAPTER 16

Potential Short-term Tests to Detect Chemicals Capable of Causing Reproductive and Developmental Dysfunction

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16.1 INTRODUCTION

The reproductive cycle encompasses a wide variety of complex interactions at the molecular, cellular and structural levels within a specific chronological sequence. The cycle starts with gametogenesis and includes all the differentiatinal and developmental processes occurring during the prenatal and postnatal periods. A broad spectrum of biological processes is represented, and includes cellular replication, tissue development and differentiation, neuroendocrine regulation, peptide and steroid hormone synthesis and action, secretory processes, and smooth muscle function, among others. Each of these processes is vulnerable to a multiplicity of toxic interferences. It is, therefore, extremely unlikely that a small number of 'simple' tests will ensure the identification of all possible adverse reproductive and developmental effects.

In recent years, a great number of short-term tests for assessing reproductive toxicity has become available and can be performed in *in vivo* and *in vitro* systems. These have been recently reviewed in Vouk and Sheehan (1983). These tests have been developed as adjuncts to the existing array of primary test batteries and are designed to: (a) rank compounds for scheduling further testing; (b) generate more specific data on the basic biological processes which are involved in the reproductive cycle; and (c) further characterize the mode(s) of action of agents known to affect essential biological processes of the reproductive cycle.

In vivo tests evaluate reproductive performance and perinatal toxicity. *In vitro* methods assess responses to test chemicals and other toxic agents (for example, ionizing and non-ionizing radiation), employing systems involving the growth and development of cells, tissues and organs of invertebrate and vertebrate origins, and whole-embryo cultures of each. As our knowledge of basic mechanisms increases, it

may also be possible to programme computer simulation models for quantitative analyses of the qualitative processes associated with particular developmental dysfunctions.

We seek to understand how foreign chemicals perturb essential biological processes and cause physiological dysfunction. Information is sought at all levels of hierarchical biological organization, but especially at the molecular level involving interaction between the exogenous chemical and its 'receptor' for toxicity. As our understanding of mechanisms of toxicity increases, laboratory testing to define toxicological hazards, and clinical examination for the identification of the earliest effect(s) of chemical exposure become more objective. In many cases, methods become more efficient, more time- and cost-effective, better conserve laboratory test animals, and involve fewer investigations. However, most short-term tests have not been validated for regulatory use.

16.2 EFFECTS ON THE MALE REPRODUCTIVE SYSTEM

In general, damage to gonads and their functions can result from: (a) direct actions of chemicals on germs cells; (b) actions affecting the accessory sex organs; and (c) actions on overall hormonal systems at either the gonadal or the hypothalamic-pituitary level. Temporally, these actions can occur either prenatally or during different time periods of postnatal life. The processes by which a chemical exerts its effects, whether through direct action, through interference with receptor interactions, or through altered steroid-metabolizing enzymes, is ultimately important for extrapolation to other chemicals or species. Evidence suggests that environmental chemicals have exerted their effects on reproduction via each of these mechanisms, often affecting more than one (World Health Organization, 1984).

16.2.1 Male accessory sex organs

The male accessory sex organs (namely, prostate, seminal vesicles and bulbourethral gland) produce secretions which serve as a buffer, vehicle or source of nutrients for sperm. In man, about 30 per cent of the semen volume comes from the prostate, while 60 per cent is produced by the seminal vesicles (Vouk and Sheehan, 1983).

Prostatic secretions are rich in acid phosphatase, lysozymes, citric acid, citric acid aminotransferase, dehydrogenases, zinc and magnesium, among other substances, while seminal vesicle secretions are rich in fructose and prostaglandins (Vouk and Sheehan, 1983). These biochemical constituents are androgen-dependent and can be measured to assess androgenic activity of toxicants, using testosterone as a reference standard.

Seminal vesicle fructose has been used as a sensitive indicator for testosterone and other androgens. It decreases following castration and can be restored by androgen administration. Fructose can be measured spectrophotometrically by several

different techniques (Thomas *et al.*, 1968). Prostate citric acid (Humphrey and Mann, 1949) and zinc (Gunn and Gould, 1956) concentrations can also be used to assess androgenic activity. These chemical indicator tests for androgens are more sensitive than gravimetric responses used in bioassay procedures (Thomas and Bell, 1982). Androgen-binding protein (ABP) can also be quantified.

Short-term *in vivo* bioassays based on the response of male sex accessory organs to androgens have been traditionally used to assess androgenic substances (Zarrow *et al.*, 1964). Newer techniques make use of radioimmunoassays (RIAs), enzyme immunosorbent assays (ELISAs), and high pressure liquid chromatography (HPLC).

16.2.2 Effects on androgen production and androgen receptors

Androgens are the principal sex hormones produced by the testis. The most important of these is testosterone secreted by the Leydig cells. The conversion of testosterone to oestrogen is thought to take place in the Sertoli cells. Although the precise hormonal regulation of spermatogenesis is not completely understood, current information suggests that testosterone is involved in such key processes as the generation of spermatogonia and the first meiotic division of diplotene cells at puberty. The androgen-protein (ABP) produced by Sertoli cells transports androgens to differentiating germ cells. The Sertoli cells have recently been the subject of much research (Mather *et al.*, 1982).

A potential testicular toxin could be studied by exposing primary cultures and continuous cell lines of rat or mouse Leydig cells to various concentrations of the toxin in the presence of luteinizing hormone (LH) and then comparing the amount of testosterone produced to that produced by LH alone. The amount of testosterone produced by the Leydig cells can be measured in the culture medium by radioimmunoassay (Furuyama *et al.*, 1970; Kinouchi *et al.*, 1973). The sensitivity of the RIA techniques continues to be refined to detect very low levels.

Androgen production and transport are regulated by the anterior pituitary gonadotropins. Follicle-stimulating hormone (FSH) stimulates Sertoli cells to produce androgen-binding protein (ABP). LH stimulates the synthesis of androgens in the interstitial cells.

Chemicals can interfere with androgen production by either directly affecting steroid synthesis in interstitial cells or by interfering with the regulation of steroid synthesis by the pituitary gonadotropins. Effects of chemicals on the production of testosterone and ABP have been studied *in vivo* and are now being investigated in *in vitro* cultures of Leydig and Sertoli cells. The responsiveness of Sertoli cells (preferably from post-pubertal males) in culture can be used to assess ABP following stimulation by FSH and incubation with the test chemical.

Cytoplasmic and nuclear androgen receptors in the target tissues are being actively studied and toxicologic applications are being increasingly sought. Gonadotropin receptors also have been demonstrated in the testes of some species.

Affinity constants of these receptors can be estimated and the effects of various exogenous chemicals on hormone–receptor interactions determined.

16.2.3 Effects on sperm

Substances can pass from blood into semen via the fluid from the excurrent ducts and the male accessory glands and could be spermicidal or alter sperm function. Such effects can be tested *in vitro* by exposing sperm (incubated in a protein-rich buffer at 37°C for 4 to 8 hours) for various periods (up to 8 hours) of time. The decline in percentage of motile sperm over time is a suitable criterion to study dose–response relationships. Other criteria, such as integrity of the acrosome and plasma membrane, oxygen consumption, adenosine 5'-triphosphate content, or degree of agglutination could be used. Methods are available for extended semen analysis, including survival and motility patterns, metabolic patterns, intracellular constituents, as well as sperm penetration of cervical mucus (Eliasson, 1978). It is felt, however, that this latter test is not a reliable indicator for predicting spermicidal action of agents *in vivo* (ORNL, 1982).

16.2.4 Biotransformation of toxicants in testicular tissues

Exogenous chemicals introduced into an organism may undergo chemical transformations resulting in metabolites which may be more toxic than the parent compound. The toxic effects of certain polycyclic hydrocarbons in tissues have been demonstrated to be due to metabolites (an epoxide) which interact with DNA, RNA and other macromolecules. The rate of epoxide formation and detoxifying enzyme activities in tissues or cells are important determinants of tissue-specific toxicity and of the sensitivity of cells to the toxicant's actions.

Appreciable activities of aryl hydrocarbon hydroxylase (AHH), epoxide hydrolase (EH), glutathione transferase (GSH-SY), and cytochrome P-450 have been found in isolated, perfused testicular tissues (Mukhtar *et al.*, 1978). The distribution of these enzymes in the interstitial and germ cell compartments indicates that AHH activity and cytochrome P-450 content of microsomes from the interstitial cells are nearly twice that in the seminiferous tubules. In contrast, the specific activity of the detoxication enzymes EH and GSH-ST in seminiferous tubules is twice that in the interstitial cells. Although AHH activity in interstitial cells has been found to be only 5 per cent that of hepatic microsomes, its close proximity to the germ cells may increase its importance with regard to the effects of enzyme-activated toxic chemicals on germ cells. However, haemodynamics and the blood–testis barrier must be considered when drug- or toxicant-metabolizing activity is determined *in vitro*.

Factors affecting induction of AHH and cytochrome P-450 may play a significant role in germ cell toxicity. Both testicular and prostatic AHH activity and cytochrome P-450 are significantly induced by 2,3,7,8-tetrachlorodibenzo-*p*-dioxin (TCDD). TCDD doubled the AHH activity in the rat testis and increased

AHH activity in the prostate 150 times (Dixon and Lee, 1980a). Thus, exposure to environmental chemicals can induce significant activation of enzymes in the testis as well as in the prostate, thereby suggesting modulation of potential germ cell toxicity. The isolation of a toxicant from specific testicular tissues and the measurement of drug-metabolizing enzyme activities in these tissues following exposure to chemicals can be used as an indicator of such effects. The velocity sedimentation technique (Lee and Dixon, 1972) can be used to separate physically complex spermatogenic cell populations into homogeneous subpopulations capable of normal function. This technique also can be used to determine the cellular affinity of specific spermatogenic cells for chemicals, including trace metals (Lee and Dixon, 1973).

16.2.5 DNA damage, synthesis, and repair in spermatogenic cells

DNA repair by spermatogenic cells is a sensitive indicator of chemically-induced DNA damage. Evidence for this form of repair has come from the detection of unscheduled DNA synthesis (UDS), sometimes called 'repair synthesis'. The uptake of ^3H -thymidine into cells that are not in S-phase is generally taken as an indicator of UDS (Dixon and Hall, 1982).

Cellular fractionation of tissue demonstrates that the uptake of thymidine label is normally confined to spermatogonial cells, which indicates that these cells are uniquely involved in normal DNA synthesis (Dixon and Lee, 1980b). Following administration of DNA-damaging chemicals (such as methyl-methanesulphonate), thymidine incorporation has been demonstrated not only in spermatogonia but also in the leptotene, zygotene, pachytene, and diplotene cells (Lee and Dixon, 1978). Because the postmeiotic cell types do not normally incorporate thymidine, they are sensitive dosimeters of DNA damage when the repair mechanism is triggered.

16.3 EFFECTS ON THE FEMALE REPRODUCTIVE SYSTEM

The ovary is responsible for two roles in reproduction: nurture and release of gametes and hormone production. Xenobiotic compounds can alter both these aspects of ovarian function. The assessment of oocyte and follicle toxicity of chemicals is of great significance because the effect is irreversible: there is no mechanism for repopulation of oocytes in the ovary.

16.3.1 Assessment of oocyte and follicle toxicity

A short-term *in vivo* murine assay has been recommended as a sensitive indicator for xenobiotic effect on oogenesis (ORNL, 1982). The test is performed on inbred mouse strains which have been shown to be the most sensitive test strains for oocyte and follicle toxicity assays (Mattison, 1979). Prenatal as well as postnatal treatments are employed. After treatment, mice are sacrificed at varying time intervals and their

ovaries removed, fixed, serially sectioned and stained. Oocytes and follicles are counted, and stages of follicular development are quantified and compared (Pedersen and Peters, 1968). The percentage of atretic follicles and the relative percentages of primordial, growing and graafian follicles are calculated. The assay appears to be a much more sensitive indicator of oocyte and follicle damage than alterations in fertility. Evidence suggests that as many as 90 per cent of all oocytes have to be destroyed before alterations in fertility of the female can be observed.

Assessment of oocyte and follicle toxicity of chemicals can also be performed *in vitro* in ovarian organ cultures by morphological assessment of follicular and oocytic growth and maturation, incidence of follicular atresia, and biochemical analysis of follicular fluid composition.

Effects of chemicals on oögonia can be evaluated *in vivo* by cytological quantitation. The test compound is administered to the experimental animal on the day of maximal oögonial proliferation. The day of sacrifice is based on the time when most of the oögonia are in dictyale stage. Gametotoxicity is assessed using indices of oögonial mitosis and germ cell degeneration. Germ cell responses to toxicants have been shown not only by a decrease in the total number of oocytes, but also by a change in the relative number of oocytes at different stages of meiosis. Similar methods utilizing *in vitro* organ cultures of fetal ovaries from humans and other mammals have also been used (Neal and Baker, 1974; Kurilo *et al.*, 1983).

16.3.2 Effects on ovarian steroid synthesis

The predominant steroids produced by the ovary are oestrogen (primarily 16 β -oestradiol), progesterone, and androgens. Follicular oestrogen is produced by direct cell secretion or granulosa cell aromatization of thecal androgen (Armstrong and Dorrington, 1977). Androgens are produced by thecal and interstitial cells. Progesterone is produced by the corpus luteum formed from both granulosa and thecal compartments after ovulation. Under the influence of the hypophyseal gonadotropins, endocrine activity of the ovary is diphasic: (i) secretion of oestrogen during the first, follicular phase; (ii) excretion of both oestrogen and progesterone produced by the corpus luteum in the second, luteal phase. A drop in both steroids occurs at the time of menses in a non-conceptive cycle. Androgens are secreted throughout a non-conceptive cycle, with a slight rise in midcycle. An alteration in steroidogenic processes can have major effects on the reproductive function.

In vitro models are valuable for assessment of the effect of toxicants on steroid synthesis because of the direct action of toxicants on the culture components. The following aspects are of importance in developing a model system: (a) the cell-type specific steroids to be measured; (b) the cycle-related variations in sex steroid production; and (c) the key biochemical rate-limiting steps in steroid biosynthesis.

Adequate methodology is currently available for isolation of ovarian cell types (Haney and Schomberg, 1978; McNatty *et al.*, 1979), tissue or organ culture, and direct RIA of media for individual steroids (Butcher *et al.*, 1974; Korenman, 1969).

Because of the ease of culture, purity of cell type, and active basal steroidogenesis, isolated granulosa cell cultures represent attractive models for evaluation of a toxicant's potential effect on steroidogenesis.

Biochemical rate-limiting steps in gonadal steroid secretion include: (a) substrate (cholesterol) availability; (b) follicle-stimulating hormone (FSH) induction of granulosa cell aromatase activity converting thecal androgens to oestrogens; and (c) luteinizing hormone (LH) induction of enzyme steps converting cholesterol to pregnenolone.

It should be noted that the removal of the cells from their approximation to the thecal layer, contact with follicular fluid, or disruption of intricate cell-to-cell contact, may alter their steroidogenic potential and morphologic appearance *in vitro*. For these reasons, the use of intact follicle cells, without separation of the thecal and granulosa compartments, may have to be considered as a test system, if problems are encountered with isolated cell systems.

To detect long-acting oestrogenic compounds with slow clearance *in vivo* such as clomiphene and nafoxidine, oestrogen receptor assays can be done *in vivo* (Clark *et al.*, 1978; 1979). These assays involve injecting various dose levels of the compound into immature rats and measuring the nuclear accumulation and cytoplasmic depletion of oestrogen receptors in the uterine tissues (Clark *et al.*, 1979). The test is sensitive, easy to perform and requires relatively few animals.

Short-term *in vivo* tests used routinely for assessment of oestrogenic activity involve determination of the time of vaginal opening and uterine epithelial cell hypertrophy in the rat after treatment with the test compound shortly after birth (Gellert *et al.*, 1972; Clark and McCormack, 1980). The tests are simple, reproducible and quite sensitive to oestrogenic compounds.

16.3.3 Receptor interactions

The study of nuclear and cytoplasmic hormone receptors (oestradiol and progesterone receptors are especially important) in target tissues is a rapidly developing field with important toxicological applications. Chemicals may compete for these receptors, alter their conformation, induce qualitative changes in their cellular content, or uncouple them from intercellular events.

The oestrogen receptor may play a role in the toxicity of many environmental agents. Metabolites of DDT, DMBA, PCBs, and similar aromatic foreign chemicals, have been reported to bind to the cytoplasmic receptors for oestrogen (Dixon and Hall, 1982). Thus, interactions between xenobiotics and cellular receptors for endogenous hormones can result in inadvertent hormonal response (agonist) or can depress normal hormonal balance (antagonist). In either case, abnormal quantitative responses by reproductive tissues can result.

A sensitive and reproducible method for *in vitro* oestrogen receptor analysis involves the use of toxicants to compete with labelled oestradiol in binding to uterine cytoplasmic oestrogen receptors (Clark *et al.*, 1979; Katzenellenbogen *et al.*,

1980; Kupfer and Bulger, 1980). This test involves the addition of various concentrations of the toxicant to uterine cytosol fractions in the presence of labelled oestradiol. If the toxicant is oestrogenic, it will compete with oestradiol for binding to receptor sites, and a classic competitive inhibition curve can be obtained. From this curve, a relative binding affinity can be calculated that reflects the agonistic or antagonistic activity of the toxicant. Such estimates of oestrogenic activity can be used to extrapolate oestrogenic activity in humans. The test is simple and requires little expense.

16.4 INTERFERENCE WITH HYPOTHALAMIC-PITUITARY FUNCTION

The gametogenic and secretory functions of the gonads are dependent upon the secretion of gonadotropins (FSH, LH) and prolactin from the anterior pituitary gland which, in turn, is controlled by the hypothalamus through gonadotropin-releasing hormones. The sex hormones feed back through the hypothalamus to inhibit gonadotropin secretion by controlling releasing factors. FSH stimulates follicular development in the ovary and spermatogenesis in the testis. LH, referred to as interstitial-cell-stimulating hormone (ICSH) in the male, causes luteinization of the ovary and stimulates androgen production by testicular Leydig cells. Prolactin is involved in the initiation and maintenance of lactation in women. In rodents, prolactin maintains the corpus luteum; it is not lutetotropic in women (Thomas and Bell, 1982).

A toxicant can adversely affect reproduction by altering the rate of secretion of one or more hormones that are synthesized and released by the hypothalamus and anterior pituitary gland. Some chemicals (for example, reserpine) act on endogenous catecholamines which, secondarily, influence the ability of the sympathetic nervous system to stimulate gonadotropin release (Coppola *et al.*, 1966; Currie *et al.*, 1969). Others, like phenothiazine derivatives, suppress the hypothalamus and bring about a decrease in gonadotropin levels by suppressing the synthesis and/or release of gonadotropin-releasing factor (Jarvik, 1965). Substances that have oestrogenic activity or act as dopamine antagonists can stimulate the secretion of prolactin and, as a consequence of the hyperprolactinaemia, gonadotropin secretion becomes suppressed.

In vitro model systems are needed for the assessment of toxicants that influence these processes. Currently, little is known about such model systems but their value for predicting toxicity is potentially great. Listed below are a few *in vitro* model systems (ORNL, 1982) which can be used to assess the effects of toxicants on the secretion of gonadotropins by pituitary cells.

16.4.1 Release of hormones from pituitary cells

The ability for a test substance to stimulate the release of gonadotropins from

pituitary cells can be measured, using pituitary cells cultured in monolayers from oestrogen–progesterone primed female rats. The gonadotropin-releasing standard should be synthetic gonadotropin-releasing hormone (GnRH). After three to five days in culture, the cells can be incubated in the presence of luteinizing hormone-releasing hormone (LHRH) or the test substance. The response parameter (production of LH, for example) can be measured in the culture medium by a radio-immunoassay (RIA) (Monroe *et al.*, 1968; Millar and Aehnelt, 1977). Using this system, a bioassay can be performed to evaluate the gonadotropin-releasing activity of the test substance and its relative potency in terms of LHRH.

Anterior pituitary tissue from female rats castrated four to six weeks before testing can be used to assay a substance for inhibition of gonadotropin release. For a reference standard, 17β -oestradiol could be used to suppress the release of LH in the culture medium.

The potential of a test substance to stimulate the release of prolactin can be measured in monolayer cultures of anterior pituitary cells obtained from young or mature female rats. The reference standard for the release of prolactin could be haloperidol. After three to five days in culture, the reference standard or the test substance is introduced and prolactin release measured by RIA. RIAs for prolactin have been developed for several species (Hwang *et al.*, 1971; Neill and Reichert, 1971; Clemons and Nicoll, 1977), and for *in vitro* bioassays (Frantz and Turkington, 1972).

To test a substance for inhibition of prolactin release, anterior pituitary tissue from oestrogenized female rats can be used under *in vitro* conditions. For a reference standard, bromoergocriptine could be used. Anterior pituitary tissue can be incubated in the presence of various concentrations of bromoergocriptine or the test substance to establish a dose–response curve. Then a bioassay can be conducted, where the concentration of prolactin in the culture medium is the responsive variable.

At present, there are no suitable model systems for assessment of agents which alter the secretion of GnRH, or the secretion of dopamine and norepinephrine by hypothalamic neurons.

16.4.2 Indirect *in vivo* assays

Levels of pituitary gonadotropins can be assessed indirectly (for example, in the female, measurement of testosterone or the histological assessment of spermatogenesis). Short-term *in vivo* methods for the bioassay of FSH and LH are available. These tests measure a quantitative response of a target organ under stimulation by the hormone or test chemical (Steelman and Pohley, 1953; Parlow, 1961). However, the bioassays of gonadotropins have been largely abandoned due to their lack of sensitivity. The sensitivity of RIAs offers substantial advantages over bioassays, even though cross-reactivity between the gonadotropins and other

hormones may weaken the correlation between biological and immunological activities.

16.5 EFFECTS ON FERTILIZATION

Many of the toxic influences on germ cells of both sexes are manifested in reduced rates of conception. There is increasing evidence from animal studies that certain chemicals can inhibit the fertilizing ability of sperm without marked effects on any of the common semen parameters (Tsunoda and Chang, 1976a,b).

The phenomenon of fertilization can be arbitrarily divided into four phases: capacitation; penetration of the egg by the sperm; activation of the egg; and the union of egg and sperm pronuclei. *In vitro* fertilization methods allow the identification of chemicals which alter the fertilization process.

16.5.1 *In vitro* heterologous fertilization tests

In vitro heterologous fertilization tests are the only means available for assessing the fertilizing capacity of human sperm. Human *in vitro* fertilization cannot itself be used as a test, therefore, substitutes are used for the human ovum. These include the zona-free hamster ova (Yanagimachi *et al.*, 1976) and the zona pellucida of stored human follicular oocytes (Yanagimachi *et al.*, 1979).

The heterologous *in vitro* fertilization technique involves a series of steps (Dixon and Hall, 1982). After performing standard semen analysis, the sperm is capacitated. Zona-free hamster ova are added to control and experimental sperm and three hours later fertilizing capacity is assayed (for the sperm binding to the ovum; decondensed sperm head; and/or the presence of the pronucleus with the corresponding sperm tail in the ovum's cytoplasm). Further normal development, such as genetic union of the cells, does not occur under these artificial conditions. A consistent failure of human sperm to penetrate more than 10 per cent of zona-free hamster eggs is considered to reflect an abnormality of the physiological events associated with fertilization (that is, sperm capacitation or the acrosome reaction).

16.5.2 *In vitro* homologous fertilization tests

Effects of chemicals on further fertilization events, for example, activation of the egg and the union of egg and sperm nuclei, can be assessed by homologous *in vitro* fertilization tests utilizing laboratory animals. Sperm penetration of the ovum, cortical reaction, decondensation of sperm nucleus, release of second polar body, appearance of male and female pronuclei, and cleavage of the early embryo are all directly analogous in laboratory animals and man. *In vitro* fertilization can also be coupled with pre-implantation embryo culture, transfer of blastocysts to pseudopregnant recipients, and evaluation of pregnancy outcome to identify critical early developmental targets of environmental chemicals. Male and female gametes

can be exposed to environmental agents either *in vitro* or *in vivo* and then be used for *in vitro* fertilization. The zygote is subsequently cultured and the early embryo transferred to a pseudopregnant recipient. Using this approach, the following parameters can be monitored: *in vitro* fertilizing capacity; 4- and 8-cell stage formation; morula and blastocyst development; implantation success, pregnancy rate, and malformations. Thus, chemical effects on the sperm and ova, early development, pre-implantation and post-implantation embryos, and birth defects can be studied.

16.6 SYSTEMS USED AS PRE-SCREENS FOR REPRODUCTIVE AND DEVELOPMENTAL DYSFUNCTION

Various developmental systems (cell and organ cultures, whole-embryo cultures, and whole-animals) have been used as supplements to standard teratology bioassays. These systems have been used for the elucidation of basic mechanisms involved in abnormal development and for the ranking of chemicals for further standard teratogenesis testing. The latter is especially important, since the number of chemical agents introduced into the environment continues to increase. Rejection of an agent on the basis of a pre-screen test would avoid the implementation of costly and time consuming standard tests.

A simpler system is preferable to the whole organism only if the underlying toxic mechanism is largely understood, occurs universally, and can be completely mimicked. In the short-term tests used in mutagenicity studies, these prerequisites seem to have been fulfilled to some extent, but even there, no single test system has been found to be fully adequate. The situation for prenatal toxicity is more complex than for mutagenicity, since numerous mechanisms are known to result in abnormal development. Thus, no one test system is likely to identify all the abnormal reactions that can occur. Short-term test methods and their applicability have been reviewed by Neubert (1983) and Shepard *et al.* (1983).

An outline of the array of developmental systems now in use is provided in Table 16.1. Those systems which have been more extensively studied and/or utilized as tools of basic research or proposed for pre-screens will be highlighted below.

16.6.1 Mammalian *in vivo* tests

A simple and inexpensive test has been developed which utilizes the tendency of fetal toxicity to manifest itself as reduced perinatal growth and/or increased perinatal death (due to either *in utero* resorptions or postnatal maternal cannibalism) (Chernoff and Kavlock, 1982). The protocol for this test involves treating the pregnant mammal during the period of organogenesis and allowing the dams to give birth. The litters are examined, counted and weighed at birth. Postnatal growth and viability have been closely correlated with effects seen after the far more laborious standard teratology bioassay (Brown and Fabro, 1981; Doe *et al.*, 1983). This *in vivo* assay maintains the feto-maternal-placental unit and can be utilized with any

Table 16.1 Short-term tests for detecting effects on fetal development

System	Period of development covered	Developmental process studied and endpoint	Reference
I. <i>Mammalian (In vivo)</i>	Organogenesis	Perinatal growth and viability	Chernoff and Kavlock (1982); Doe <i>et al.</i> (1983)
II. <i>Mammalian (whole-embryo culture)</i>			
Mammalian embryos (including human)	Pre-implantation	Fertilization, cleavage, and blastocyst formation	Brinster (1970); Spielmann and Eibs (1978); Hsu (1980)
Rodent embryos	Post-implantation organogenesis	Organogenesis over 2 (or 3) days	New (1966a, b); Cockcroft and Coppola (1977); Shepard <i>et al.</i> (1969); Chatot <i>et al.</i> (1980); Klee-Trieschmann and Neubert (1981); Sadler and Warner (1984); Priscott <i>et al.</i> (1984)
III. <i>Organ culture</i>			
Limb buds (avian, rodent)	Late organogenesis	Morphogenesis, cartilage formation, muscle formation	Kochhar (1975); Neubert <i>et al.</i> (1974); Neubert and Bluth (1981)
Human digits		Pharmacological effects and growth	Rajan (1969); Rajan <i>et al.</i> (1980)
Palatal shelves	Organogenesis	Epithelial fusion and cell death, palate closure	Lahti and Saxén (1967); Saxén (1973); Pratt (1938)
Lens	Organogenesis	Lens differentiation, histogenesis, and protein production	Karkinen-Jaaskelainen <i>et al.</i> (1975)
Sex organs	Late organogenesis	Gonadal and organ development, germ cell maturation, accessory sex gland histogenesis	Jost and Bergerard (1949); Josso (1974); Lasnitzki and Mizuno (1981)

Kidney	Late organogenesis	Nephrogenesis, histogenesis	Crocker (1973); Saxén and Ekblom (1981)
Thyroid	Late organogenesis	Thyroid histogenesis functional development	Shepard (1974)
IV. <i>Tissue culture</i>			
Lung	Organogenesis	Pattern formation, cell recognition, and adhesion	Merker <i>et al.</i> (1981)
Skeletal muscle	Organogenesis	Myogenesis, cell fusion, and histogenesis	Holtzer <i>et al.</i> (1958)
Teratocarcinoma	Organogenesis	Histogenesis	Stevens (1967)
Human embryonic cells	Organogenesis	Growth of palatal and mesenchymal cells	Pratt (1983); Pratt <i>et al.</i> (1982); Wilk <i>et al.</i> (1980)
Neural cells	Organogenesis	Histogenesis	Flint (1983); Flint <i>et al.</i> (1984)
V. <i>Sub-mammalian systems</i>			
Chick embryo (<i>in ovo</i>)	Entire	Whole development	Jelinek (1982); Jelinek and Peterka (1981); Jelinek and Rychter (1979)
Amphibian embryos	Entire	Whole development	Schultz <i>et al.</i> (1982)
Fish embryos	Entire	Whole development	Streisinger (1975)
<i>Hydra attenuata</i>	Adult and embryonic	Aggregation and movement, regeneration of damaged and dissociated adults	Johnson (1980); Johnson <i>et al.</i> (1982)
Sea urchins	Cleavage	Cell replication and contacts	Costello <i>et al.</i> (1957); Hagstrom and Lonning (1973)
<i>Drosophila spp.</i>	Embryogenesis	Embryogenesis	Buzin and Bournias-Vardiabasis (1984)

applicable route of exposure. It has an additional advantage over standard tests in that it can enable investigators to identify postnatal function deficits that may not be apparent in prenatal morphological end-points.

16.6.2 Mammalian embryo cultures

It is now possible to culture mammalian embryos from the one- or two-cell stage to the blastocyst stage. In general, pre-implantation embryos cannot be cultured beyond stages corresponding to implantation or early post-implantation. Hsu (1979) has developed a system in which it is possible to study mammalian development *in vitro* from the pre-implantation phase to the late embryonic stages. However, the success rate of this model has not yet exceeded 10 per cent. Rat embryos (8.5–10.5 day-old) can be cultured over a 48-hour period, and embryonic stages corresponding to about 35-somites can be reached (Fantel, 1982; Sadler *et al.*, 1982).

Some studies have been conducted on mouse (Davis *et al.*, 1981) and hamster embryos (Givelber and Di Paolo, 1968). Rat embryos have been cultured traditionally in a medium containing rat serum, but some investigators have successfully grown them in human serum (Chatot *et al.*, 1980; Klein *et al.*, 1980), dialysed serum (Gunberg, 1976), and in a chemically-defined, serum-free medium (Klee-Trieschmann and Neubert, 1981).

Popov *et al.* (1981), Fantel *et al.* (1979), and others, have used a liver S9 microsomal system added to the culture to activate cyclophosphamide. Oglesby *et al.* (1984) have described an embryo culture system in which rat embryos are grown in the presence of hepatocytes of alternative species by allowing hepatic metabolites of one species to affect development of the other. Only gross morphological evaluations have been performed in most of this study. A morphological scoring system (Brown and Fabro, 1981) may be useful for evaluation but, for more detailed analysis, histological examination is essential (Herken and Anschuetz, 1981).

The applicability of the whole-embryo technique as a routine method is limited because it is expensive, too sensitive to disturbances, often of unknown origin, and prone to abnormal development even under normal conditions. Furthermore, a maximum culture period of 48 hours is too short compared with the entire period of organogenesis.

The ultimate utility of mammalian embryo culture may lie in the ability to subject laboratory animal embryos to human tissue metabolites where appropriate heterologous systems are developed. Testing of compounds that are insoluble or soluble in toxic solvents is difficult and false positives are common with surface-active compounds.

16.6.3 Chick embryos *in ovo* used as pre-screens

Chemicals have frequently been tested in chick embryos at all stages of development by administration into fertile eggs (Fisher and Schoenwolf, 1983). This ap-

proach has not always provided results comparable with those obtained in mammalian systems. Moreover, it is doubtful whether the form and concentration of a chemical, when it reaches the embryo, can be known. A standard window technique for delivering the chemical to the chick embryo has been developed for embryotoxicity and teratogenicity studies in which better control of experimental conditions is possible (Jelinek and Peterka, 1981). The test substance is administered in a single application to the subgerminal portion of the yolk of the embryo on day 2, and intra-amniotically on days 3 or 4. The number of dead, malformed and growth-retarded embryos are counted on day 8 of incubation, when the study is terminated. The method is inexpensive and requires a moderate degree of skill. One of the major problems centres on the fact that the greatly reduced metabolic activity and difference in biochemical composition of the avian egg compared with a mammalian embryo can make the form and concentration of the xenobiotic very different.

16.6.4 Vertebrate embryo cultures

Test systems utilizing the embryos of amphibia and fish have been proposed for teratology screens (Dumont *et al.*, 1983; Streisinger, 1975). These tests are difficult to perform with water-insoluble compounds. Their potential use as screens remains unknown.

16.6.5 Invertebrates used as pre-screens

A number of invertebrates have been proposed as potential teratology pre-screens. These include *Drosophila* (Schuler *et al.*, 1982); *Planaria* (Best and Morita, 1982); and *Hydra* (Johnson, 1980; Johnson *et al.*, 1982). The respective end-points are developmental throughout entire metamorphosis; regeneration; and reassociation of disassociated adult cells. Should these systems be validated, they could be useful for rapid and inexpensive screening of large numbers of chemicals. However, the phylogenetic distance from humans may limit their usefulness for extrapolation.

16.6.6 Organ cultures

Two approaches to organ culture have been used: (a) the fetal organ is maintained on a filter or other support, and differentiates at the culture medium–gas interface (Trowell, 1961; and (b) the fetal organ is submerged in the culture medium and rotated to facilitate gas exchange and diffusion.

The Trowell technique is used for the culture of limb buds from rat (Shepard and Bass, 1970) or mouse (Aydelotte and Kochhar, 1972) embryos. It can also be used for limb buds from embryos of other species, for example, rabbit, chick, or ferret (Lessmollmann *et al.*, 1976; Beck and Gulamhusein, 1980), in a serum-containing medium or a chemically-defined medium. Growth of limb buds in culture is retarded

compared with *in vivo* development, but morphological differentiation of the cartilaginous bone anlagen and muscle can be observed. The extent of the morphogenetic differentiation of the cartilaginous bone anlagen depends largely on the stage of development at which the cultures are initiated.

The submerged culture system has also been used successfully for the study of limb bud differentiation *in vitro* (Neubert and Barrach, 1977; Blankenburg *et al.*, 1981). Abnormal development has been induced *in vitro* by adding various teratogens to the culture medium (Welsch *et al.*, 1978; Stahlmann *et al.*, 1981).

It has been possible to quantify the extent of differentiation achieved in culture and to establish dose-response relationships for abnormal development using the organ culture system. Biochemical variables, such as levels of DNA, RNA, protein, collagen, etc., can be measured (Neubert *et al.*, 1974); or a score system may be used for morphogenetic differentiation (Neubert *et al.*, 1977a,b; 1978).

Growth and differentiation of the palatal shelves have been achieved in organ culture. This system has been used extensively to study cleft palate-inducing agents (Lahti and Saxen, 1967; Pratt, 1983).

Lens development can be followed using human embryonic material. The induction of abnormal development by rubella virus can be mimicked *in vitro*. Cataract formation was observed by Karkinen-Jaaskelainen *et al.* (1975).

Organ culture techniques have been developed for the study of explanted mouse or human embryonic kidney (Crocker, 1973; Saxén and Ekblom, 1981). This system may prove useful for investigating the normal mechanism of renal development and also for testing suspected nephrotoxic agents. The expense and time involved are both fairly high since the end-points require histological analysis.

Organ culture of embryonic gonads together with accessory ducts has contributed to the understanding of how hormones and some hormone inhibitors modify sexual development.

16.6.7 Tissue culture systems

Primary cultures from various embryonic tissues have been used in developmental studies, including the chick neural crest (Greenberg, 1982), heart mesenchymal cells, and skeletal muscle cells (Holtzer *et al.*, 1958). However, it must be remembered that some cells may change their characteristics in culture. Pratt *et al.* (1982) have used an established cell line from the human embryonic palatal mesenchyme (HEPM) to study the biochemical basis for the way in which glucocorticoids inhibit palatal mesenchymal cell growth and cause cleft palate in the rodent.

It seems possible that, in some instances, primary cell cultures of embryonic or fetal cells, including those from human embryos or fetuses, can be used for studies on the drug-metabolizing capacity of cells during prenatal, and especially perinatal, development (Nau *et al.*, 1977; Egger *et al.*, 1978; Liddiard *et al.*, 1978; Merker *et al.*, 1981; Kremers *et al.*, 1981).

16.6.8 Studies with non-embryonic tissues

There are a number of non-embryonic tissue systems which can be used to study mechanisms of embryotoxic effects. Basic processes, such as cell-cell interactions, cell migrations, differentiation processes, proliferation processes, and cell death, can be used as end-points, as these are involved in embryonic development. Systems used include the investigation of aggregation phenomena with mammalian cells (Moscona and Moscona, 1952), cell-cell interactions as studied by the attachment of cells to lectin-coated surfaces (Braun *et al.*, 1982), and the inhibition of mammalian cell growth (Freese, 1982). The use of teratocarcinoma cells, which are pluripotent cells capable of many types of embryonic differentiation, might also be considered (Stevens, 1967; Graham, 1977; Martin, 1978).

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