Document

No 33

'ENVIRONMENTAL OESTROGENS':

A Compendium of Test Methods

July 1996

'ENVIRONMENTAL OESTROGENS':

A Compendium of Test Methods

ECETOC DOCUMENT No. 33

July 1996

ECETOC TASK FORCE ON 'ENVIRONMENTAL OESTROGENS'

'ENVIRONMENTAL OESTROGENS' : A COMPENDIUM OF TEST METHODS

Preamble

Against a background of increasing concern about the potential for natural and synthetic chemicals to affect reproduction in humans and wildlife, there is a pressing need to develop screening and testing methods which will enable an unequivocal identification of such hazardous properties. Experimental data from a variety of laboratory studies are appearing at an increasing rate, providing a dilemma to those involved in hazard and risk assessment because the reliability of the methods has yet to be demonstrated.

The Task Force is in the process of evaluating the utility of available test methods for assessing hazards to humans and wildlife. As a first step, a compendium of methods which have been reported to identify chemicals affecting reproductive function (including those having oestrogenic activity) has been compiled. It is published at this stage to encourage others to provide information to add to the compendium and to effect the critical review of the methods.

In publishing this compendium, the Task Force is aware of the limitations of such methods for use in regulatory toxicology; for example, there are acknowledged limitations in using *in vitro* methods because they may fail to account for the complex metabolic and kinetic interactions known to be important to the expression of toxicity in whole animals. Similarly *in vivo* studies may have limitations in that functional effects may occur as a result of a variety of mechanistic interactions and may not be relevant to other species. Such aspects have a profound influence on the utility of methods for evaluating chemical risks, hence, the listing of a method in this compendium does not imply that it is necessarily of value for such purpose.

TABLE 1: - REGULATORY TEST METHODS - MAMMALIAN *in vivo* * (Commonly Used Species)

Method	Principle	Endocrine/reproductive endpoint(s) determined	Reference
Subchronic Studies (Rats, Mice, Dogs)	Repeated administration of the test substance for a period of 28 days or 90 days.	- Endocrine and sex accessory organ weights and appearance - Histopathology of endocrine or sex accessory organs	1,2,3,4,5,6, 7,8,9,10,11
Long-term Studies (Rats, Mice, Dogs)	Repeated administration of the test substance for a period of 12 months for dogs, 18 months in mice, and 24 months in rats.	- Endocrine and sex accessory organ weights and appearance - Histopathology of endocrine or sex accessory organs	12,13,14,15, 16,17,18
Multi-generation Reproduction and/or Continuous Breeding Studies (Rats, Mice)	Repeated administration of the test substance during the entire reproductive cycle of males and females.	Reproductive function, capacity, and fecundity for 1 or more generations and/or continuous breeding - Altered function and morphology of endocrine or sex accessory organs of parental animals or their offspring - All aspects of reproductive physiology (i.e., insemination and fertility rates, perinatal development and overall fecundity) New proposed OPPTS 870.3800 will incorporate - measurement of sperm count, motility, and morphology - oestrous cycle length and normality - developmental landmarks such as vaginal opening and preputial separation - detailed histopathology of ovaries, testes, and epididymides	19,20,21,22, 23,24,25
ICH (International Conference on Harmonisation) Reproductive and Developmental Toxicology Studies	Repeated administration " Most Probable Options" screens to evaluate fertility, embryonic, pre- and post-natal development, and maternal function. Secondary repeated administration tests are designed to characterise the mechanism and origin of any toxic effects.	- Maturation of gametes (spermato- and oocyto-genesis) - Mating behaviour - Fertility and fecundity - Pre- and post-implantation embryo development - *Corpora lutea,* implantation sites - Live and dead conceptus - Abnormalities and malformation of fetuses - Postnatal growth, development, and mortality of the offspring - Functional deficits in the offspring (behaviour, maturation, and reproduction) - Altered function and morphology (macro- and micro-) of the endocrine and sex accessory organs of parental animals and their offspring.	24

* See Annex 1 for more details

TABLE 1: (Cont.) REGULATORY TEST METHODS - MAMMALIAN *in vivo* * (Commonly Used Species)

Method	Principle	Endocrine/reproductive endpoint(s) determined	Reference
Prenatal Developmental Toxicity Studies (Rats, Mice, Rabbits) [Formerly Called Teratology or Segment II]	Single or repeated administration during prenatal development of the fetuses of pregnant animals exposed during critical period of organogenesis. The new OPPTS 870.3700 guideline will incorporate exposure from implantation until term.	- Implantation, abortion, early embryonic death, and resorption - External and internal malformation or anomaly rates	24,25,26,27, 28,29,30,31, 32
Reproduction / Developmental Toxicity Screening Test (OECD 421) (Rats)	This reduced one-generation study is designed to generate limited information concerning the effects of a chemical on male and female reproductive performance. The testing is initiated 2 weeks prior to mating and continued until day 4 postnatally.	- Gonadal function - Mating behavior - Stages of spermatogenesis - Conception - Development of the conceptus - Parturition - Early postnatal survival and growth	33
Combined Repeated Dose Toxicity Study with the Reproduction/ Developmental Toxicity Screening Test (Draft OECD 422) (Rats)	Combined repeated dose toxicity study with a reduced one-generation study is designed to provide information on the effects of repeated exposure on the health and reproductive performance of both sexes.	- Endocrine and sex accessory organ weights and appearance - Histopathology of endocrine or sex accessory organs - Gonadal function - Mating behaviour - Conception - Development of the conceptus - Parturition - Early postnatal survival and growth	34
Dominant Lethal Test (Mice, Rats)	Repeat mating or repeat dosing with 1 to 3 matings to evaluate chemical mutagenesis in germ cell in adult males.	- Effects on different germ cell stages - Indirect evidence on spermatogenesis - Information on sperm fertility if coupled to ova analysis	35,36

* See Annex 1 for more details

TABLE 2: NON-REGULATORY TEST METHODS - MAMMALIAN *in vivo*

Method	Principle	Endocrine/reproductive endpoint(s) determined	Reference
In vivo Developmental Toxicity Screening Tests	Administration to pregnant rats or mice from day 8 through 12 of gestation. Effects on progeny studied.	- Growth retardation, reduced viability, stillbirths, malformations, offspring viability and growth reduction	37
Chernoff-Kavlock	Range finding non-pregnant rodents followed by dosing day 8-12 of pregnancy with Minimal Toxic Dose.	- Potential female reproductive effects - Potential fetal/postnatal growth and survival, in general, directs further studies	38
Allen-Doisy Assay	Cornification and keratinization of vaginal epithelium of ovariectomized rodents.	- Vaginal cytology	39,40,41,42, 43
Vaginal Epithelial Cell Proliferation	Increase in vaginal mitosis and epithelial thickness in ovariectomized rodents.	- Alteration in vaginal epithelial mitotic index	42,43,44,45, 46,47
Vaginal Epithelial Tetrazolium Reduction	Increase in vaginal reduction of tetrazolium in ovariectomized rodents measured colorimetrically.	- Tetrzolöium reduction	48
Vaginal Opening	The opening of the vagina in the immature rodent.	- Precocious or delayed vaginal opening - Reproductive tract weight	49,50,51
Vaginotrophic Response	Increase in vaginal wet weight in the immature female rodent.	- Vaginal tissue growth response	52,53,54,55
Measurement of Sialinacid	Oestrogen-induced reduction of vaginal sialinacid production in ovariectomized female rodents.	- Reduction in vaginal sialinacid production	56
Uterine Growth Test	Increase in the weight of the uterus (wet and/or dry) of ovariectomized or immature rodents.	- Change in uterine weight	53,54,55
Uterine Fluid Inbibition (Astwood Bioassay)	Specialised uterine growth test measuring early water uptake.	- Dose response increase in uterine wet weight	57,58,59

TABLE 2: (Cont.) NON-REGULATORY TEST METHODS - MAMMALIAN *in vivo*

Method	Principle	Endocrine/reproductive endpoint(s) determined	Reference
Uterine Growth Biochemistry	Measurements of biochemical events associated with uterine growth in ovariectomized or immature rodents.	- Thymidine incorporation into DNA - Glycogen content in response to estrogens - Enzyme activity - Increased peroxidase activity	59,60,61,62 63,64
Uterine Estrogen Withdrawal Bleeding	In women and non-human primates, sudden deprivation or diminution of oestrogen or progestin results in uterine bleeding.	- Induced uterine bleeding	65,66,67
Serum and/or Tissue Hormone Levels measured using Radioimmunoassay	Exposure to oestrogens or oestrogenic agents can alter normal hormonal balance in intact animals; changes in specific hormones can be measured as a direct or indirect measure of oestrogenic potential.	- Serum and/or tissue hormone concentrations	68,69,70,71, 72

TABLE 3: NON-REGULATORY TEST METHODS - MAMMALIAN *in vitro*

Method	Principle	Endocrine/reproductive endpoint(s) determined	Reference
In vitro Developmental Toxicity Study	The determination of the effects of chemicals on micromass.	- Differentiation towards cartilage and neuronal cells - Growth retardation, cellular toxicity and death	73
Chick Embryo Retina Cell Assay	Evaluates the teratogenic potential embryonic cells.	- Cell to cell interaction - Growth and differentiation in embryonic cells	74,75
Whole - Embryo Culture System	Evaluates the teratogenic potential *in vitro.*	- Altered growth or differentiation of embryonic cells	76,77,78,99
MCF - 7 Cell Proliferation *In Vitro* (E-Screen Assay)	Cell proliferation in a polyclonal, hormonally responsive cultured human metastatic breast cancer cell line (MCF-7).	- Relative proliferative potencies (RPP) - Variations of this approach use other endpoints, i.e., Cathepsin-D release, thymidine incorporation, tetrazolium, Alamar Blue reduction and progesterone receptor concentations.	79,80,81,82
Ishikawa Cell (Human Endometrial Carcinoma Line) - Alkaline Phosphatase	Measurement of alkaline phosphatase release in response to oestrogen.	- Measurement of alkaline phosphatase release	83,84
Oestrogen/Androgen Receptor Binding	Oestrogen/Androgen reacts with a high binding affinity and specificity to protein which constitutes a primary event in hormonal action. These receptor proteins are located in the primary as well as secondary accessory organs.	- Displacement of oestradiol or testosterone from isolated receptor protein	85,86
Aryl Hydrocarbon Receptor (Ah) Binding (Dioxin Receptor)	Although the Ah receptor is not the oestrogen receptor, experimental evidence would suggest some relationship between the two receptor types.	- Measurement of binding to the Ah receptor	87,88,89
Uterine cell DNA and Progesterone Receptor Synthesis/Specific Protein Secretion	Dispersed uterine cells from an immature rat grown in monoculture are oestrogen responsive.	- Induction of progesterone receptor - Induction of 130-Kdalton secreted protein - Thymidine incorporation	90,91

TABLE 3: (Cont.) NON-REGULATORY TEST METHODS - MAMMALIAN *in vitro*

Method	Principle	Endocrine/reproductive endpoint(s) determined	Reference
Pituitary Cell Prolactin Synthesis	Primary cultures of dispersed rat pituitary cells from immature rats respond to estrogen by increased incorporation of leucine into prolactin.	- Incorporation of leucine into prolactin	92,93
In Vitro - Transfected Cells	The oestrogen receptor functions by modulating the rate of transcription of its target cell genes. The receptor contains at least two Transcriptional Activation Functions (TAFs), TAF-1 is located at the N terminal end to the DNA binding domain while TAF-2 is located at the C terminus of the hormone binding domain. The relative activities of the TAFs vary according to exposure to oestradiol and other oestrogens. Measurement of the specific receptor - chemical interactions, linking specific endocrine-responsive elements to CAT or Luciferase protein genes in human and animal cell lines that are responsive to specific hormones or hormone -mimics, is possible with transfected cells.	- Characterisation of receptor-chemical interaction and potency by means of measuring CAT or Luciferase	94,95,96,97
In vitro Fertilization Assay Using Zona Pellucida Free Hamster, Rat, or Mouse eggs	Evaluation of fertilization in eggs with the *zona pellucida* removed	- Penetration by spermatozoa - Cell division	98
In vitro Fertilization in Cultured Embryos	Evaluation of the mammalian preimplantation period in cultured embryos	- Cell division from first cleavage through implantation - Toxicity to meiosis - Chromosomal effects	99
Relative Binding Affinity of Serum Hormone Binding Proteins	The effect of serum (i.e. binding proteins) on the binding affinity to the oestrogen receptor.	- Relative binding of the test substance in the presence and absence of serum proteins	100
Maintenance of Female Reproductive Organ Cell in Culture.	Oestrogen is needed for the maintenance of ovarian somatic cells (granulosa, thecal, and stromal cell) in culture.	- Effects on cell morphology - Effects on cell viability - Hormonal responsiveness	101

TABLE 3: (Cont.) NON-REGULATORY TEST METHODS - MAMMALIAN *in vitro*

Method	Principle	Endocrine/reproductive endpoint(s) determined	Reference
Microtubule Polymerization Assay in Syrian Hamster Embryos	Inhibition of microtubule polymerization leading to micronuclei and aneuploidy, and perhaps estrogen-mediated carcinogenicity.	- Microtubule polymerization in a cell free system	102
Mouse Lactoferrin Gene as a Marker for Oestrogen and Epidermal Growth Factor	Two modules on the mouse uterus lactoferrin gene respond to the presence of oestrogen.	- Measurement of lactoferrin mRNA and protein	103

TABLE 4: REGULATORY TEST METHODS - ENVIRONMENTAL *in vivo* *

Method	Principle	Endocrine/reproductive endpoint(s) determined	Reference
Fish Early Life Stage Tests	Commences with the exposure of fertilized fish eggs and continues through to juvenile stages.	- Hatchability and viability of eggs, - Growth and development of fish larvae, including abnormal behaviour and appearance	104
Fish Partial Chronic Test	Commences with the exposure of adults and continues through to the first generation early life stage.	- Fecundity of adult fish (frequency and hatching rate of eggs, survival of embryos and larvae) - Alteration in hatching	105
Fish Full Life-Cycle Test	Commences with the exposure of eggs and continues through to 8 weeks post-hatch in the second generation.	- Viability of eggs - Development - Growth - Reproductive success	105
Invertebrate Reproduction Tests (e.g. *Daphnia magna*, *Chironomus*, Mysid shrimp, annelids)	Commences with exposure of neonates/juveniles continuing through to adult reproduction.	- Detection of toxic effects on reproduction	106, 107,108, 109
Avian Tests	Adult Mallard duck or Japanese quail are exposed *via* the diet (20 wks) through to egg hatch and young birds monitored for 14 days.	- Mortality of adults - Egg production - Cracked eggs and egg shell thickness - Viability, - Hatchability - Effects on young birds	110,111,112

* See Annex 1 for more details

TABLE 5: NON-REGULATORY TEST METHODS - ENVIRONMENTAL *in vivo*

Method	Principle	Endocrine/reproductive endpoint(s) determined	Reference
Mollusca	Exposure of juveniles or adults and observation of morphological changes.	- Changes to sexual characteristics - Imposex - Sexual differentiation - Reproduction	113,114,115, 116,117
Amphibia	Commences with exposure of amphibian tadpoles continuing through to the adulthood.	- Change in the phenotypic sex ratio - Plasma vitellogenin concentration	118,119
Reptilia	Turtle and alligator eggs are exposed by painting or injecting the test substance (estrogenic activity, aromatase inhibition). Vitellogenin induction in Red-eared turtles.	- Changes in hatchling sex ratios - Plasma vitellogenin concentration	119,120,121, 122, 123
Fish-Early Life Stage Tests	Commences with the exposure of fertilized fish eggs and continues through to juvenile stages.	- Abnormal gonad differentiation - Alteration of phenotypic sex ratio - Presence of intersex fish	124
Fish-Partial Chronic Test	Commences with the exposure of adult fish and continues through to the first generation early life stage.	- Alteration in hatching frequency and rate of eggs - Survival of embryos and larvae	125
Fish - Vitellogenin Synthesis in Immature or Male Fish	Exposure of juvenile or adult male fish and vitellogenin production in response to oestrogen.	- Plasma vitellogenin concentration	126,127,128, 129
Fish-Reproductive Effects in Maturing or Mature Fish.	Exposure of immature or mature fish.	- Abnormal expression of secondary sexual characteristics - Regression of gonads, - Change in plasma steroid concentration - Alteration of *in vitro* gonadal steroidogenesis	130

TABLE 5: (Cont.) NON-REGULATORY TEST METHODS - ENVIRONMENTAL *in vivo*

Method	Principle	Endocrine/reproductive endpoint(s) determined	Reference
Fish - 2-Generation Chronic Test.	Exposure of fish over 2 generations.	- Reduction in eggs released per spawning	131,132
Avian	Exposure of chicken eggs to endocrine modulators (aromatase inhibition).	- Change in hatchling sex ratios	133

TABLE 6: NON-REGULATORY TEST METHODS - ENVIRONMENTAL *in vitro*

Method	Principle	Endocrine/reproductive endpoint(s) determined	Reference
Receptor Binding (fish liver)	Binding to reproductive steroid receptors.	- Concentration of xenobiotics required to cause a 50% displacement of steroid (e.g. [3H]- oestradiol)	134
Recombinant Yeast Cell Assay	Oestrogen- and androgen-inducible expression system and transcriptional activity.	- Reporter gene activity (galactosidase)	135,136,137
Transfected Estrogen Receptor, Chicken Embryo Fibroblasts	Oestrogen-induced transcriptional activity.	- Reporter gene activity (chloramphenolacetyl transferase and luciferase)	134
Vitellogenin Synthesis by Fish Hepatocytes	Vitellogenin production in response to oestrogen stimulation.	- Vitellogenin concentration	125