Adopted:
22nd January 2001

OECD GUIDELINE FOR THE TESTING OF CHEMICALS

PROPOSAL FOR UPDATING GUIDELINE 414

Prenatal Developmental Toxicity Study

INTRODUCTION

1. In Copenhagen in June 1995, an OECD Working Group on Reproduction and Developmental Toxicity discussed the need to update existing OECD Test Guidelines for reproduction and developmental toxicity and the development of new Guidelines for endpoints not yet covered. The Working Group recommended that the Guideline for Developmental Toxicity should be revised, based on a proposal received from the US (1). The Working Group reached agreement on all major elements of the revised version of this Guideline.

INITIAL CONSIDERATIONS

2. This guideline for developmental toxicity testing is designed to provide general information concerning the effects of prenatal exposure on the pregnant test animal and on the developing organism; this may include assessment of maternal effects as well as death, structural abnormalities, or altered growth in the foetus. Functional deficits, although an important part of development, are not a part of this Guideline. They may be tested for in a separate study or as an adjunct to this study using the Guideline for developmental neurotoxicity. For information on testing for functional deficiencies and other postnatal effects the Guidelines for the two-generation reproductive toxicity study and the developmental neurotoxicity study should be consulted.

3. This guideline may require specific adaptation in individual cases on the basis of specific knowledge on e.g. physicochemical or toxicological properties of the test substance. Such adaptation is acceptable, when convincing scientific evidence suggests that the adaptation will lead to a more informative test. In such a case, this scientific evidence should be carefully documented in the study report.

4. Definitions used are given in the Annex.

PRINCIPLE OF THE TEST

5. Normally, the test substance is administered to pregnant animals at least from implantation to one day prior to the day of scheduled kill, which should be as close as possible to the normal day of delivery without risking loss of data resulting from early delivery. The guideline is not intended to examine solely the period of organogenesis, (e.g. days 5-15 in the rodent, and days 6-18 in the rabbit) but also effects from preimplantation, when appropriate, through the entire period of gestation to the day before caesarean section. Shortly before

caesarean section, the females are killed, the uterine contents are examined, and the foetuses are evaluated for soft tissue and skeletal changes.

PREPARATION FOR THE TEST

Selection of animal species

6. It is recommended that testing be performed in the most relevant species, and that laboratory species and strains which are commonly used in prenatal developmental toxicity testing be employed. The preferred rodent species is the rat and the preferred non-rodent species is the rabbit. Justification should be provided if another species is used.

Housing and feeding conditions

7. The temperature in the experimental animal room should be 22 (±3 °C) for rodents and 18 (±3 °C) rabbits. Although the relative humidity should be at least 30% and preferably not exceed 70% other than during room cleaning, the aim should be 50-60%. Lighting should be artificial, the sequence being 12 hours light, 12 hours dark. For feeding, conventional laboratory diets may be used with an unlimited supply of drinking water.

8. Mating procedures should be carried out in cages suitable for the purpose. While individual housing of mated animals is preferred, group housing in small numbers is also acceptable.

Preparation of the animals

9. Healthy animals, which have been acclimated to laboratory conditions for at least 5 days and have not been subjected to previous experimental procedures, should be used. The test animals should be characterised as to species, strain, source, sex, weight and/or age. The animals of all test groups should, as nearly as practicable, be of uniform weight and age**.** Young adult nulliparous female animals should be used at each dose level. The females should be mated with males of the same species and strain, and the mating of siblings should be avoided. For rodents day 0 of gestation is the day on which a vaginal plug and/or sperm are observed; for rabbits day 0 is usually the day of coitus or of artificial insemination, if this technique is used. Mated females should be assigned in an unbiased manner to the control and treatment groups. Cages should be arranged in such a way that possible effects due to cage placement are minimised. Each animal should be assigned a unique identification number. Mated females should be assigned in an unbiased manner to the control and treatment groups, and if the females are mated in batches, the animals in each batch should be evenly distributed across the groups. Similarly, females inseminated by the same male should be evenly P

PROCEDURE

Number and sex of animals

10. Each test and control group should contain a sufficient number of females to result in approximately 20 female animals with implantation sites at necropsy. Groups with fewer than 16 animals with implantation sites may be inappropriate. Maternal mortality does not necessarily invalidate the study providing it does not exceed approximately 10 percent.

Preparation of doses

11. If a vehicle or other additive is used to facilitate dosing, consideration should be given to the following characteristics: effects on the absorption, distribution, metabolism, and retention or excretion of the test substance; effects on the chemical properties of the test substance which may alter its toxic characteristics; and effects on the food or water consumption or the nutritional status of the animals. The vehicle should neither be developmentally toxic nor have effects on reproduction.

Dosage

12. Normally, the test substance should be administered daily from implantation (e.g., day 5 post mating) to the day prior to scheduled caesarean section. If preliminary studies, when available, do not indicate a high potential for preimplantation loss, treatment may be extended to include the entire period of gestation, from mating to the day prior to scheduled kill. It is well known that inappropriate handling or stress during pregnancy can result in prenatal loss. To guard against foetal loss from factors which are not treatment-related, unnecessary handling of pregnant animals as well as stress from outside factors such as noise should be avoided.

13. At least three dose levels and a concurrent control should be used. Healthy animals should be assigned in an unbiased manner to the control and treatment groups. The dose levels should be spaced to produce a gradation of toxic effects. Unless limited by the physical/chemical nature or biological properties of the test substance, the highest dose should be chosen with the aim to induce some developmental and/or maternal toxicity (clinical signs or a decrease in body weight) but not death or severe suffering. At least one intermediate dose level should produce minimal observable toxic effects. The lowest dose level should not produce any evidence of either maternal or developmental toxicity. A descending sequence of dose levels should be selected with a view to demonstrating any dosage-related response and no-observed-adverse-effect level (NOAEL) or doses near the limit of detection that would allow the determination of a benchmark dose. Two- to four-fold intervals are frequently optimal for setting the descending dose levels, and the addition of a fourth test group is often preferable to using very large intervals (e.g. more than a factor of 10) between dosages. Although establishment of a maternal NOAEL is the goal, studies which do not establish such a level may also be acceptable (2).

14. Dose levels should be selected taking into account any existing toxicity data as well as additional information on metabolism and toxicokinetics of the test substance or related materials. This information will also assist in demonstrating the adequacy of the dosing regimen.

15. A concurrent control group should be used. This group should be a sham-treated control group or a vehicle-control group if a vehicle is used in administering the test substance. All groups should be administered the same volume of either test substance or vehicle. Animals in the control group(s) should be handled in an identical manner to test group animals. Vehicle control groups should receive the vehicle in the highest amount used (as in the lowest treatment group).

Limit test

16. If a test at one dose level of at least 1000 mg/kg body weight/day by oral administration, using the procedures described for this study, produces no observable toxicity and if an effect would not be expected based upon existing data (e.g., from structurally and/or metabolically related compounds), then a full study

using three dose levels may not be considered necessary. Expected human exposure may indicate the need for a higher oral dose level to be used in the limit test. For other types of administration, such as inhalation or dermal application, the physical chemical properties of the test substance often may indicate the maximum attainable level of exposure (for example, dermal application should not cause severe localised toxicity).

Administration of doses

17. The test substance or vehicle is usually administered orally by intubation. If another route of administration is used, the tester should provide justification and reasoning for its selection, and appropriate modifications may be necessary (3)(4)(5). The test substance should be administered at approximately the same time each day.

18. The dose to each animal should normally be based on the most recent individual body weight determination. However, caution should be exercised when adjusting the dose during the last trimester of pregnancy. Existing data should be used for dose selection to prevent excess maternal toxicity. However, if excess toxicity is noted in the treated dams, those animals should be humanely killed. If several pregnant animals show signs of excess toxicity, consideration should be given to terminating that dose group. When the substance is administered by gavage, this should preferably be given as a single dose to the animals using a stomach tube or a suitable intubation canula. The maximum volume of liquid that can be administered at one time depends on the size of the test animal. The volume should not exceed 1 ml/100 g body weight, except in the case of aqueous solutions where 2 ml/100 g body weight may be used. When corn oil is used as a vehicle, the volume should not exceed 0.4 ml/100 g body weight. Variability in test volume should be minimised by adjusting the concentrations to ensure a constant volume across all dose levels.

Observations of the dams

19. Clinical observations should be made and recorded at least once a day, preferably at the same time(s) each day taking into consideration the peak period of anticipated effects after dosing. The condition of the animals should be recorded including mortality, moribundity, pertinent behavioural changes, and all signs of overt toxicity.

Body weight and food consumption.

20. Animals should be weighed on day 0 or no later than day 3 if time-mated animals are supplied by an outside breeder, on the first day of dosing, at least every 3 days during the dosing period and on the day of scheduled kill.

21. Food consumption should be recorded at three-day intervals and should coincide with days of body weight determination.

Post-mortem examination

22. Females should be killed one day prior to the expected day of delivery. Females showing signs of abortion or premature delivery prior to scheduled kill should be killed and subjected to a thorough macroscopic examination.

23. At the time of termination or death during the study, the dam should be examined macroscopically for any structural abnormalities or pathological changes**.** Evaluation of the dams during caesarean section and

subsequent foetal analyses should be conducted preferably without knowledge of treatment group in order to minimise bias.

Examination of uterine contents

24. Immediately after termination or as soon as possible after death, the uteri should be removed and the pregnancy status of the animals ascertained. Uteri that appear non-gravid should be further examined (e.g. by ammonium sulphide staining for rodents and Salewski staining or a suitable alternative method for rabbits) to confirm the non-pregnant status (6).

25. Gravid uteri including the cervix should be weighed. Gravid uterine weights should not be obtained from animals found dead during the study.

26. The number of corpora lutea should be determined for pregnant animals.

27. The uterine contents should be examined for numbers of embryonic or foetal deaths and viable foetuses. The degree of resorption should be described in order to estimate the relative time of death of the conceptus (see Annex for definitions).

Examination of foetuses

28. The sex and body weight of each foetus should be determined.

29. Each foetus should be examined for external alterations (7).

30. Foetuses should be examined for skeletal and soft tissue alterations (e.g. variations and malformations or anomalies) (8)(9)(10)(11)(12)(13)(14)(15)(16)(17)(18)(19)(20)(21)(22)(23)(24)(25). Categorisation of foetal alterations is preferable but not required. When categorisation is done, the criteria for defining each category should be clearly stated. Particular attention should be paid to the reproductive tract which should be examined for signs of altered development.

31. For rodents, approximately one-half of each litter should be prepared and examined for skeletal alterations. The remainder should be prepared and examined for soft tissue alterations, using accepted or appropriate serial sectioning methods or careful gross dissection techniques.

32. For non-rodents, e.g. rabbits, all foetuses should be examined for both soft tissue and skeletal alterations. The bodies of these foetuses are evaluated by careful dissection for soft tissue alterations, which may include procedures to further evaluate internal cardiac structure (26). The heads of one-half of the foetuses examined in this manner should be removed and processed for evaluation of soft tissue alterations (including eyes, brain, nasal passages and tongue), using standard serial sectioning methods (27) or an equally sensitive method. The bodies of these foetuses and the remaining intact foetuses should be processed and examined for skeletal alterations, utilising the same methods as described for rodents.

DATA AND REPORTING

Data

33. Data shall be reported individually and summarised in tabular form, showing for each test group and each generation the number of animals at the start of the test, the number of animals found dead during the test

or killed for humane reasons, the time of any death or humane kill, the number of pregnant females, the number of animals showing signs of toxicity, a description of the signs of toxicity observed, including time of onset, duration, and severity of any toxic effects, the types of foetal observations, and all relevant litter data.

34. Numerical results should be evaluated by an appropriate statistical method using the litter as the unit for data analysis. A generally accepted statistical method should be used; the statistical methods should be selected as part of the design of the study. Data from animals that do not survive to the scheduled kill should also be reported. These data may be included in group means where relevant. Relevance of the data from such an animal, and therefore inclusion or exclusion from any group mean(s), should be judged on an individual basis.

Evaluation of Results

35. The findings of the Prenatal Developmental Toxicity Study should be evaluated in terms of the observed effects. The evaluation will include the following information:

- maternal and foetal test results, including an evaluation of the relationship, or lack thereof, between the exposure of the animals to the test substance and the incidence and severity of all findings;
- criteria used for categorising foetal external, soft tissue, and skeletal alterations if categorisation has been done;
- when appropriate, historical control data to enhance interpretation of study results;
- the numbers used in calculating all percentages or indices;
- adequate statistical analysis of the study findings, when appropriate, which should include sufficient information on the method of analysis, so that an independent reviewer/statistician can re-evaluate and reconstruct the analysis.

36. In any study which demonstrates an absence of toxic effects, further investigation to establish absorption and bioavailability of the test substance should be considered.

Test report

37. The test report must include the following specific information:

Test substance:

- physical nature and, where relevant, physiochemical properties;
- identification including CAS number if known/established;
- purity.

Vehicle (if appropriate):

- justification for choice of vehicle, if other than water.

Test animals:

- species and strain used;
- number and age of animals;

- source, housing conditions, diet, etc.;
- individual weights of animals at the start of the test.

Test conditions:

- rationale for dose level selection;
- details of test substance formulation/diet preparation, achieved concentration, stability and homogeneity of the preparation;
- details of the administration of the test substance;
- conversion from diet/drinking water test substance concentration (ppm) to the actual dose (mg/kg body weight/day), if applicable;
- environmental conditions;
- details of food and water quality.

Results:

- Maternal toxic response data by dose, including but not limited to:

- the number of animals at the start of the test, the number of animals surviving, the number pregnant, and the number aborting, number of animals delivering early;
- day of death during the study or whether animals survived to termination;
- data from animals that do not survive to the scheduled kill should be reported but not included in the inter-group statistical comparisons;
- day of observation of each abnormal clinical sign and its subsequent course;
- body weight, body weight change and gravid uterine weight, including, optionally, body weight change corrected for gravid uterine weight;
- food consumption and, if measured, water consumption;
- necropsy findings, including uterine weight;
- NOAEL values for maternal and developmental effects should be reported.

Developmental endpoints by dose for litters with implants, including:

- number of corpora lutea;
- number of implantations, number and percent of live and dead foetuses and resorptions;
- number and percent of pre- and post-implantation losses.

Developmental endpoints by dose for litters with live foetuses, including:

- number and percent of live offspring;
- sex ratio;
- foetal body weight, preferably by sex and with sexes combined;
- external, soft tissue, and skeletal malformations and other relevant alterations;
- criteria for categorisation if appropriate;
- total number and percent of foetuses and litters with any external, soft tissue, or skeletal alteration, as well as the types and incidences of individual anomalies and other relevant alterations.

Discussion of results.

Conclusions.

Interpretation of Results

38. A prenatal developmental toxicity study will provide information on the effects of repeated oral exposure to a substance during pregnancy. The results of the study should be interpreted in conjunction with the findings of subchronic, reproduction, toxicokinetic and other studies. Since emphasis is placed on both general toxicity and developmental toxicity endpoints, the results of the study will allow for the discrimination between developmental effects occurring in the absence of general toxicity and those which are only expressed at levels that are also toxic to the maternal animal (28).

LITERATURE

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ANNEX

Developmental toxicology: the study of adverse effects on the developing organism that may result from exposure prior to conception, during prenatal development, or postnatally to the time of sexual maturation. The major manifestations of developmental toxicity include 1) death of the organism, 2) structural abnormality, 3) altered growth, and 4) functional deficiency. Developmental toxicology was formerly often referred to as teratology.

Adverse effect: any treatment-related alteration from baseline that diminishes an organism's ability to survive, reproduce or adapt to the environment. Concerning developmental toxicology, taken in its widest sense it includes any effect which interferes with normal development of the conceptus, both before and after birth.

Altered growth: an alteration in offspring organ or body weight or size.

Alterations (anomalies): structural alterations in development that include both malformations and variations (29):

> **Malformation**/Major Abnormality: Structural change considered detrimental to the animal (may also be lethal) and is usually rare.
> **Variation**/Minor Abnormality Structural change considered to have little or no detrimental effect on the animal; may be transient and may occur relatively frequently in the control population.

Conceptus: the sum of derivatives of a fertilised ovum at any stage of development from fertilisation until birth including the extra-embryonic membranes as well as the embryo or foetus.

Implantation (nidation): attachment of the blastocyst to the epithelial lining of the uterus, including its penetration through the uterine epithelium, and its embedding in the endometrium.

Embryo: the early or developing stage of any organism, especially the developing product of fertilisation of an egg after the long axis appears and until all major structures are present.

Embryotoxicity: detrimental to the normal structure, development, growth, and/or viability of an embryo.

Foetus: the unborn offspring in the post-embryonic period.

Foetotoxicity: detrimental to the normal structure, development, growth, and/or viability of a foetus.

Abortion: the premature expulsion from the uterus of the products of conception: of the embryo or of a nonviable foetus.

Resorption: a conceptus which, having implanted in the uterus, subsequently died and is being, or has been resorbed:

Early resorption: evidence of implantation without recognisable embryo/foetus.
Late resorption: dead embryo or foetus with external degenerative changes.

NOAEL: abbreviation for no-observed-adverse-effect level and is the highest dose level where no adverse treatment-related findings are observed.