Section 4
Health effects

Test Guideline No. 422

Combined Repeated Dose Toxicity Study with the Reproduction/Developmental Toxicity Screening Test

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OECD Guidelines for the Testing of Chemicals

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OECD GUIDELINES FOR THE TESTING OF CHEMICALS

Combined Repeated Dose Toxicity Study with the Reproduction/Developmental Toxicity Screening Test

INTRODUCTION

1. OECD Guidelines for the Testing of Chemicals are periodically reviewed in the light of scientific progress. The original screening Test Guideline 422 was adopted in 1996, based on a protocol for a "Combined Repeat Dose and Reproductive/Developmental Screening Test" discussed in two expert meetings, in London in 1990 (1) and in Tokyo in 1992 (2).

2. It combines a reproduction/developmental toxicity screening part which is based on experience gained in Member countries from using the original method on existing high production volume chemicals and in exploratory tests with positive control substances (3) (4), and a repeated dose toxicity part, in concordance with Test Guideline 407.

3. This TG has been updated with endocrine disruptor relevant endpoints, as a follow up to the high-priority activity initiated at OECD in 1998 to revise existing Test Guidelines and to develop new Test Guidelines for the screening and testing of potential endocrine disruptors (5). In this context TG 407 (Repeated Dose 28-Day Oral Toxicity Study in Rodents) was enhanced in 2008 by parameters suitable to detect endocrine activity of test chemicals. The objective in updating TG 422 was to include some endocrine disruptor relevant endpoints in screening TGs where the exposure periods cover some of the sensitive periods during development (pre- or early postnatal periods).

4. 4The selected additional endocrine disrupter relevant endpoints, also part of TG 443 (Extended One Generation Reproductive Toxicity Study), were included in TG 422 based on a feasibility study addressing scientific and technical questions related to their inclusion, as well as possible adaptations of the test design needed for their inclusion (6).

5. This Guideline is designed to generate limited information concerning the effects of a test chemical on male and female reproductive performance such as gonadal function, mating behaviour, conception, development of the conceptus and parturition. It is not an alternative to, nor does it replace the existing Test Guidelines 414, 415, 416 or 443.

INITIAL CONSIDERATIONS

6. 6. In the assessment and evaluation of the toxic characteristics of a test chemical the determination of oral toxicity using repeated doses may be carried out after the initial information on toxicity has been obtained by acute testing. This study provides information on the possible health hazards likely to arise from repeated exposure over a relatively limited period of time. The method comprises the basic repeated dose toxicity study that may be used for chemicals on which a 90-day study is not warranted (e.g. when the production volume does not exceed certain limits) or as a preliminary study to a long-term study. In conducting the study, the guiding principles and considerations outlined in the OECD Guidance Document n° 19 on the recognition, assessment, and use of clinical signs as humane endpoints for experimental animals used in safety evaluations (7) should be followed.

7. It further comprises a reproduction/developmental toxicity screening test and, therefore, can also be used to provide initial information on possible effects on male and female reproductive performance such as gonadal function, mating behaviour, conception, development of the conceptus and parturition, either at an early stage of assessing the toxicological properties of test chemicals, or on test chemicals of concern. This test does not provide complete information on all aspects of reproduction and development. In particular, it offers only limited means of detecting postnatal manifestations of prenatal exposure, or effects that may be induced during postnatal exposure. Due (amongst other reasons) to the selectivity of the end points, and the short duration of the study, this method will not provide evidence for definite claims of no reproduction/developmental effects. Moreover, in the absence of data from other reproduction/developmental toxicity tests, positive results are useful for initial hazard assessment and contribute to decisions with respect to the necessity and timing of additional testing.

8. The results obtained by the endocrine related parameters should be seen in the context of the "OECD Conceptual Framework for Testing and Assessment of Endocrine Disrupting Chemicals" (8). In this Conceptual Framework, the enhanced TG 422 is contained in level 4 as an in vivo assay providing data on adverse effects on endocrine relevant endpoints. An endocrine signal might not however be considered sufficient evidence on its own that the test chemical is an endocrine disruptor.

9. The Guideline also places emphasis on neurological effects as a specific endpoint, and the need for careful clinical observations of the animals, so as to obtain as much information as possible, is stressed. The method should identify chemicals with neurotoxic potential, and which may warrant further in-depth investigation of this aspect. In addition, the method may also give a basic indication of immunological effects.

10. In the absence of data from other systemic toxicity, reproduction/developmental toxicity, neurotoxicity and/or immunotoxicity studies, positive results are useful for initial hazard assessment and contribute to decisions with respect to the necessity and timing of additional testing. The test may be particularly useful as part of the Screening Information Data Set (SIDS) for the assessment of existing chemicals for which little or no toxicological information is available and can serve as an alternative to conducting two separate tests for repeated dose toxicity (Guideline 407) and reproduction/developmental toxicity (Guideline 421), respectively. It can also be used as a dose range finding study for more extensive reproduction/developmental studies, or when otherwise considered relevant.

11. Generally, it is assumed that there are differences in sensitivity between pregnant and non-pregnant animals. Consequently, it may be more complicated to determine dose levels in this combined test that are adequate to evaluate both general systemic toxicity and specific reproduction/developmental toxicity, rather than when the individual tests are conducted separately. Moreover, interpretation of the test results with respect to general systemic toxicity may be more difficult than when conducting a separate repeated-dose study, especially when serum and histopathology parameters are not evaluated at the same time in the study. Because of these technical complexities, considerable experience in toxicity testing is required for the performance of this combined screening test. On the other hand, apart from the smaller

number of animals involved, the combined test may offer a better means of discriminating direct effects on reproduction/development from those that are secondary to other (systemic) effects.

12. In this test, the dosing period is longer than in a conventional 28-day repeated dose study. However, it uses fewer animals of each sex per group when compared with the situation where a conventional 28-day repeated dose study is conducted in addition to a Reproduction/Developmental Toxicity Screening Test.

13. This Test Guideline has been updated to include the opportunity (optional) to collect sample tissues for cryopreservation in view of further investigations. Decisions on whether to include the optional parameters set out in this test guideline should reflect existing knowledge for the test chemical or similar chemicals, as well as the needs of various regulatory authorities.

14. This Guideline assumes oral administration of the test chemical. Modifications may be required if other routes of exposure are used.

15. Before use of the Test Guideline on a mixture for generating data for an intended regulatory purpose, it should be considered whether, and if so why, it may provide adequate results for that purpose. Such considerations are not needed, when there is a regulatory requirement for testing of the mixture.

16. Definitions used are given in Annex 1.

PRINCIPLE OF THE TEST

17. The test chemical is administered in graduated doses to several groups of males and females. Males should be dosed for a minimum of four weeks, up to and including the day before scheduled kill (this includes a minimum of two weeks prior to mating, during the mating period and, approximately, two weeks post mating). In view of the limited pre-mating dosing period in males, fertility may not be a particularly sensitive indicator of testicular toxicity. Therefore, a detailed histological examination of the testes is essential. The combination of a pre-mating dosing period of two weeks and subsequent mating/fertility observations with an overall dosing period of at least four weeks, followed by detailed histopathology of the male gonads, is considered sufficient to enable detection of the majority of effects on male fertility and spermatogenesis.

18. Females should be dosed throughout the study. This includes two weeks prior to mating (with the objective of covering at least two complete oestrous cycles), the variable time to conception, the duration of pregnancy and at least thirteen days after delivery, up to and including the day before scheduled kill.

19. Duration of study, following acclimatisation and pre-dosing oestrous cycle evaluation, is dependent on the female performance and is approximately 63 days, [at least 14 days pre-mating, (up to) 14 days mating, 22 days gestation, 13 days lactation].

20. During the period of administration, the animals are observed closely each day for signs of toxicity. Animals which die or are killed during the test are necropsied and, at the conclusion of the test, surviving animals are killed and necropsied.

DESCRIPTION OF THE METHOD

Selection of animal species

21. This Test Guideline is designed for use with the rat. If the parameters specified within this TG 422 are investigated in another rodent species a detailed justification should be given. In the international

validation program for the detection of endocrine disrupters on TG 407, the rat was the only species used. Strains with low fecundity or well-known high incidence of developmental defects should not be used. Healthy virgin animals, not subjected to previous experimental procedures, should be used. The test animals should be characterised as to species, strain, sex, weight and age. At the commencement of the study the weight variation of animals used should be minimal and not exceed ± 20% of the mean weight of each sex. Where the study is conducted as a preliminary study to a long-term or a full-generation study, it is preferable that animals from the same strain and source are used in both studies.

Housing and feeding

22. All procedures should conform to local standards of laboratory animal care. The temperature in the experimental animal room should be 22 °C (± 3°). The relative humidity should be at least 30% and preferably not exceed 70% other than during room cleaning. Lighting should be artificial, the photoperiod being 12 hours light, 12 hours dark. For feeding, conventional laboratory diets may be used with an unlimited supply of drinking water. The choice of diet may be influenced by the need to ensure a suitable admixture of a test chemical when administered by this method.

23. Animals should be group housed in small groups of the same sex; animals may be housed individually if scientifically justified. For group caging, no more than five animals should be housed per cage. Mating procedures should be carried out in cages suitable for the purpose. Pregnant females should be caged individually and provided with nesting materials. Lactating females will be caged individually with their offspring.

24. The feed should be regularly analysed for contaminants. A sample of the diet should be retained until finalisation of the report.

Preparation of the animals

25. Healthy young adult animals are randomised and assigned to the treatment groups and cages. Cages should be arranged in such a way that possible effects due to cage placements are minimised. The animals are uniquely identified and kept in their cages for at least five days prior to the start of the study to allow for acclimatisation to the laboratory conditions.

Preparation of doses

26. It is recommended that the test chemical be administered orally unless other routes of administration are considered more appropriate. When the oral route is selected, the test chemical is usually administered by gavage; however, alternatively, test chemicals may also be administered via the diet or drinking water.

27. Where necessary, the test chemical is dissolved or suspended in a suitable vehicle. It is recommended that, wherever possible, the use of an aqueous solution/suspension be considered first, followed by consideration of a solution/suspension in oil (e.g. corn oil) and then by possible solution in other vehicles. For non-aqueous vehicles the toxic characteristics of the vehicle should be known. The stability and homogeneity of the test chemical in the vehicle should be determined.

PROCEDURE

Number and sex of animals

28. It is recommended that each group be started with at least 10 males and 12-13 females. Females will be evaluated pre-exposure for oestrous cyclicity and animals that fail to exhibit typical 4-5 day cycles will not be included in the study; therefore, extra females are recommended in order to yield 10 females per group. Except in the case of marked toxic effects, it is expected that this will provide at least 8 pregnant

females per group which normally is the minimum acceptable number of pregnant females per group. The objective is to produce enough pregnancies and offspring to assure a meaningful evaluation of the potential of the test chemical to affect fertility, pregnancy, maternal and suckling behaviour, and growth and development of the F1 offspring from conception to day 13 post-partum. If interim kills are planned, the number should be increased by the number of animals scheduled to be killed before the completion of the study. Consideration should be given to an additional satellite group of five animals per sex in the control and the top dose group for observation of reversibility, persistence or delayed occurrence of systemic toxic effects, for at least 14 days post treatment. Animals of the satellite groups will not be mated and, consequently, are not used for the assessment of reproduction/developmental toxicity.

Dosage

29. Generally, at least three test groups and a control group should be used. If there are no suitable general toxicity data available, a range finding study may (animals of the same strain and source) be performed to aid the determination of the doses to be used. Except for treatment with the test chemical, animals in the control group should be handled in an identical manner to the test group subjects. If a vehicle is used in administering the test chemical, the control group should receive the vehicle in the highest volume used.

30. Dose levels should be selected taking into account any existing toxicity and (toxico-) kinetic data available. It should also be taken into account that there may be differences in sensitivity between pregnant and non-pregnant animals. The highest dose level should be chosen with the aim of inducing toxic effects but not death nor obvious suffering. Thereafter, a descending sequence of dose levels should be selected with a view to demonstrating any dosage related response and no adverse effects at the lowest dose level. Two- to four- fold intervals are frequently optimum and addition of a fourth test group is often preferable to using very large intervals (e.g. more than a factor of 10) between dosages.

31. In the presence of observed general toxicity (e.g. reduced body weight, liver , heart, lung or kidney effects, etc.) or other changes that may not be toxic responses (e.g. reduced food intake, liver enlargement), observed effects on endocrine sensitive endpoints should be interpreted with caution.

Limit test

32. If an oral study at one dose level of at least 1000 mg/kg body weight/day or, for dietary administration, an equivalent percentage in the diet, or drinking water (based upon body weight determinations), using the procedures described for this study, produces no observable toxic effects and if toxicity would not be expected based upon data from structurally related substances, then a full study using several dose levels may not be considered necessary. The limit test applies except when human exposure indicates the need for a higher dose level to be used. For other types of administration, such as inhalation or dermal application, the physical chemical properties of the test chemicals often may dictate the maximum attainable exposure.

Administration of doses

33. The animals are dosed with the test chemical daily for 7 days a week. When the test chemical is administered by gavage, this should be done in a single dose to the animals using a stomach tube or a suitable intubation cannula. 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. Except for irritating or corrosive test chemicals which will normally reveal exacerbated effects with higher concentrations, variability in test volume should be minimized by adjusting the concentration to ensure a constant volume at all dose levels.

34. For test chemicals administered via the diet or drinking water, it is important to ensure that the quantities of the test chemical involved do not interfere with normal nutrition or water balance. When the test chemical is administered in the diet either a constant dietary concentration (ppm) or a constant dose level in terms of the animals' body weight may be used; the alternative used should be specified. For a test chemical administered by gavage, the dose should be given at similar times each day, and adjusted at least weekly to maintain a constant dose level in terms of animal body weight. Where the combined study is used as a preliminary to a long term or a full reproduction toxicity study, a similar diet should be used in both studies.

Experimental schedule

35. Dosing of both sexes should begin 2 weeks prior to mating, after they have been acclimatised for at least five days and females have been screened for normal oestrous cycles (in a 2 weeks pre-treatment period). The study should be scheduled in such a way that oestrous cycle evaluation begins soon after the animals have attained full sexual maturity. This may vary slightly for different strains of rats in different laboratories, e.g. Sprague Dawley rats 10 weeks of age, Wistar rats about 12 weeks of age. Dams with offspring should be killed on day 13 post-partum, or shortly thereafter. In order to allow for overnight fasting of dams prior to blood collection (if this option is preferred), dams and their offspring need not necessarily be killed on the same day. The day of birth (viz. when parturition is complete) is defined as day 0 post-partum. Females showing no-evidence of copulation are killed 24-26 days after the last day of the mating period. Dosing is continued in both sexes during the mating period. Males should further be dosed after the mating period at least until the minimum total dosing period of 28 days has been completed. They are then killed, or, alternatively, are retained and continued to be dosed for the possible conduction of a second mating if considered appropriate.

36. Daily dosing of the parental females should continue throughout pregnancy and at least up to, and including, day 13 post-partum or the day before sacrifice. For studies where the test chemical is administered by inhalation or by the dermal route, dosing should be continued at least up to, and including, day 19 of gestation, and dosing should be re-initiated as soon as possible and not later than PND 4.

37. Animals in a satellite group scheduled for follow-up observations, if included, are not mated. They should be kept at least for a further 14 days after the first scheduled kill of dams, without treatment to detect delayed occurrence, or persistence of, or recovery from toxic effects.

38. A diagram of the experimental schedule is given in Annex 2.

Oestrous cycles

39. Oestrous cycles should be monitored before treatment starts to select for the study females with regular cyclicity (see paragraph 28). Vaginal smears should also be monitored daily from the beginning of the treatment period until evidence of mating. If there is concern about acute stress effects that could alter estrous cycles with the initiation of dosing, laboratories can expose test animals for 2 weeks, then collect vaginal smears daily to monitor estrous cycle for a minimum of two weeks during the pre-mating period with continued monitoring into the mating period until there is evidence of mating. When obtaining vaginal/cervical cells, care should be taken to avoid disturbance of mucosa, which could induce pseudopregnancy (8) (9).

Mating procedure

40. Normally, 1:1 (one male to one female) matings should be used in this study. Exceptions can arise in the case of occasional deaths of males. The female should be placed with the same male until evidence of copulation is observed or two weeks have elapsed. Each morning the females should be examined for the presence of sperm or a vaginal plug. Day 0 of pregnancy is defined as the day on which mating

evidence is confirmed (a vaginal plug or sperm is found). In case pairing was unsuccessful, re-mating of females with proven males of the same group could be considered.

Litter size

41. On day 4 after birth, the size of each litter may be adjusted by eliminating extra pups by random selection to yield, as nearly as possible, four or five pups per sex per litter depending on the normal litter size in the strain of rats used. Blood samples should be collected from two of the surplus pups, pooled, and used for determination of serum T4 levels Selective elimination of pups, e.g. based upon body weight, or anogenital distance (AGD) is not appropriate. Whenever the number of male or female pups prevents having four or five of each sex per litter, partial adjustment (for example, six males and four females) is acceptable. No pups will be eliminated when litter size will drop below the culling target (8 or 10 pups/litter). If there is only one pup available above the culling target, only one pup will be eliminated and used for blood collection for possible serum T4 assessments.

42. If litter size is not adjusted, two pups per litter are sacrificed on day 4 after birth and blood samples are taken for measurement of serum thyroid hormone concentrations. If possible the two pups per litter should be female pups to reserve male pups for nipple retention evaluations, except in the event that removing these pups leaves no remaining females for assessment at termination. No pups will be eliminated when litter size will drop below 8 or 10 pups/litter (depending on the normal litter size in the strain of rats used). If there is only one pup available above the normal litter size, only one pup will be eliminated and used for blood collection for possible serum T4 assessments.

Observations

43. General clinical observations should be made at least once a day, preferably at the same time(s) each day and considering the peak period of anticipated effects after dosing. The health condition of the animals should be recorded. At least twice daily all animals are observed for morbidity and mortality.

44. Once before the first exposure (to allow for within-subject comparisons), and at least once a week thereafter, detailed clinical observations should be made in all parental animals. These observations should be made outside the home cage in a standard arena and preferably at the same time, each day. They should be carefully recorded; preferably using scoring systems, explicitly defined by the testing laboratory. Effort should be made to ensure that variations in the test conditions are minimal and that observations are preferably conducted by observers unaware of the treatment. Signs noted should include, but not be limited to, changes in skin, fur, eyes, mucous membranes, occurrence of secretions and excretions and autonomic activity (e.g. lacrimation, piloerection, pupil size, unusual respiratory pattern). Changes in gait, posture and response to handling as well as the presence of clonic or tonic movements, stereotypies (e.g. excessive grooming, repetitive circling), difficult or prolonged parturition or bizarre behaviour (e.g. self-mutilation, walking backwards) should also be recorded (10).

45. At one time during the study, sensory reactivity to stimuli of different modalities (e.g. auditory, visual and proprioceptive stimuli) (8) (9) (11), assessment of grip strength (12) and motor activity assessment (13) should be conducted in five males and five females, randomly selected from each group. Further details of the procedures that could be followed are given in the respective references. However, alternative procedures than those referenced could also be used. In males, these functional observations should be made towards the end of their dosing period, shortly before scheduled kill but before blood sampling for haematology or clinical chemistry (see paragraphs 54-57, including footnote 1). Females should be in a physiologically similar state during these functional tests and should preferably be tested once during the last week of lactation (e.g., LD 6-13), shortly before scheduled kill. To the extent possible, minimize dams and pups separation times.

46. Functional observations made once towards the end of the study may be omitted when the study is conducted as a preliminary study to a subsequent subchronic (90-day) or long-term study. In that case, the functional observations should be included in this follow-up study. On the other hand, the availability of data on functional observations from this repeated dose study may enhance the ability to select dose levels for a subsequent subchronic or long-term study.

47. As an exception, functional observations may also be omitted for groups that otherwise reveal signs of toxicity to an extent that would significantly interfere with the functional test performance.

48. The duration of gestation should be recorded and is calculated from day 0 of pregnancy. Each litter should be examined as soon as possible after delivery to establish the number and sex of pups, stillbirths, live births, runts (pups that are significantly smaller than corresponding control pups), and the presence of gross abnormalities.

49. Live pups should be counted and sexed and litters weighed within 24 hours of parturition (day 0 or 1 post-partum) and at least on day 4 and day 13 post-partum. In addition to the observations on parent animals (see paragraphs 44 and 45), any abnormal behaviour of the offspring should be recorded.

50. The AGD of each pup should be measured on the same postnatal day between PND 0 through PND 4. Pup body weight should be collected on the day the AGD is measured and the AGD should be normalized to a measure of pup size, preferably the cube root of body weight (14). The number of nipples/areolae in male pups should be counted on PND 12 or 13 as recommended in OECD GD 151 (15).

Body weight and food/water consumption

51. Males and females should be weighed on the first day of dosing, at least weekly thereafter, and at termination. During pregnancy, females should be weighed on days 0, 7, 14 and 20 and within 24 hours of parturition (day 0 or 1 post-partum), and at least day 4 and day 13 post-partum. These observations should be reported individually for each adult animal.

52. During pre-mating, pregnancy and lactation, food consumption should be measured at least weekly. The measurement of food consumption during mating is optional. Water consumption during these periods should also be measured, when the test chemical is administered by that medium.

Haematology

53. Once during the study, the following haematological examinations should be made in five males and five females randomly selected from each group: haematocrit, haemoglobin concentrations, erythrocyte count, reticulocytes, total and differential leucocyte count, platelet count and a measure of blood clotting time/potential. Other determinations that should be carried out, if the test chemical or its putative metabolites have or are suspected to have oxidising properties include methaemoglobin concentration and Heinz bodies.

54. Blood samples should be taken from a named site. Females should be in a physiologically similar state during sampling. In order to avoid practical difficulties related to the variability in the onset of gestation, blood collection in females may be done at the end of the pre-mating period as an alternative to sampling just prior to, or as part of, the procedure for euthanasia of the animals. Blood samples of males should preferably be taken just prior to, or as part of, the procedure for euthanasia of the animals. Alternatively, blood collection in males may also be done at the end of the pre-mating period when this time point was preferred for females.

55. Blood samples should be stored under appropriate conditions.

Clinical biochemistry

56. Clinical biochemistry determinations to investigate major toxic effects in tissues and, specifically, effects on kidney and liver, should be performed on blood samples obtained from the selected five males and five females of each group. Overnight fasting of the animals prior to blood sampling is recommended( ). Investigations of plasma or serum should include sodium, potassium, glucose, total cholesterol, urea, creatinine, total protein and albumin, at least two enzymes indicative of hepatocellular effects (such as alanin aminotransferase, aspartate aminotransferase and sorbitol dehydrogenase) and bile acids. Measurements of additional enzymes (of hepatic or other origin) and bilirubin may provide useful information under certain circumstances.

57. Blood samples from a defined site are taken based on the following schedule:

- from at least two pups per litter on day 4 after birth, if the number of pups allows (see paragraphs 41-42)
- from all dams and at least two pups per litter at termination on day 13, and
- from all adult males, at termination

All blood samples are stored under appropriate conditions. Blood samples from the day 13 pups and the adult males are assessed for serum levels for thyroid hormones (T4). Further assessment of T4 in blood samples from the dams and day 4 pups is done if relevant. As an option, other hormones may be measured if relevant. Pup blood can be pooled by litter for thyroid hormone analyses. Thyroid hormones (T4 and TSH) should preferably be measured as ‘total’.

58. Optionally, the following urinalysis determinations could be performed in five randomly selected males of each group during the last week of the study using timed urine volume collection; appearance, volume, osmolality or specific gravity, pH, protein, glucose and blood/blood cells.

59. In addition, studies to investigate serum markers of general tissue damage should be considered. Other determinations that should be carried out if the known properties of the test chemical may, or are suspected to, affect related metabolic profiles include calcium, phosphate, fasting triglycerides and fasting glucose, specific hormones, methaemoglobin and cholinesterase. These need to be identified on a case-by-case basis.

60. The following factors may influence the variability and the absolute concentrations of the hormone determinations:

– time of sacrifice because of diurnal variation of hormone concentrations
– method of sacrifice to avoid undue stress to the animals that may affect hormone concentrations
– test kits for hormone determinations that may differ by their standard curves.

61. Plasma samples specifically intended for hormone determination should be obtained at a comparable time of the day. The numerical values obtained when analysing hormone concentrations differ with various commercial assay kits.

62. If historical baseline data are inadequate, consideration should be given to determination of haematological and clinical biochemistry variables before dosing commences or preferably in a set of animals not included in the experimental groups. For females, the data have to be from lactating animals.

PATHOLOGY

Gross necropsy

63. All adult animals in the study should be subjected to a full, detailed gross necropsy which includes careful examination of the external surface of the body, all orifices, and the cranial, thoracic and abdominal cavities and their contents. Special attention should be paid to the organs of the reproductive system. The number of implantation sites should be recorded. Vaginal smears should be examined on the day of necropsy to determine the stage of the oestrous cycle and allow correlation with histopathology of female reproductive organs.

64. The testes and epididymides as well as prostate and seminal vesicles with coagulating glands as a whole of all male adult animals should be trimmed of any adherent tissue, as appropriate, and their wet weight taken as soon as possible after dissection to avoid drying. In addition, optional organ weights could include levator ani plus bulbocavernosus muscle complex, Cowper's glands) and glans penis in males and paired ovaries (wet weight) and uterus (including cervix) in females; if included, these weights should be collected as soon as possible after dissection. . The ovaries, testes, epididymides, accessory sex organs, and all organs showing macroscopic lesions of all adult animals, should be preserved.

65. From all adult males and females and one male and female day 13 pup from each litter thyroid glands should be preserved in the most appropriate fixation medium for the intended subsequent histopathological examination. The thyroid weight could be determined after fixation. Trimming should also be done very carefully and only after fixation to avoid tissue damage. Tissue damage could compromise histopathology analysis. Blood samples should be taken from a named site just prior to or as part of the procedure for euthanasia of the animals, and stored under appropriate conditions (see paragraph 57).

66. In addition, for a least five adult males and females, randomly selected from each group (apart from those found moribund and/or euthanised prior to the termination of the study), the liver, kidneys, adrenals, thymus, spleen, brain and heart should be trimmed of any adherent tissue, as appropriate and their wet weight taken as soon as possible after dissection to avoid drying. The following tissues should be preserved in the most appropriate fixation medium for both the type of tissue and the intended subsequent histopathological examination : all gross lesions, brain (representative regions including cerebrum, cerebellum and pons), spinal cord, eye, stomach, small and large intestines (including Peyer's patches), liver, kidneys, adrenals, spleen, heart, thymus, trachea and lungs (preserved by inflation with fixative and then immersion), gonads (testis and ovaries), accessory sex organs (uterus and cervix, epididymides, prostate, seminal vesicles plus coagulating glands), vagina, urinary bladder, lymph nodes (besides the most proximal draining node, another lymph node should be taken according to the laboratory's experience (16)), peripheral nerve (sciatic or tibial) preferably in close proximity to the muscle, skeletal muscle and bone, with bone marrow (section or, alternatively, a fresh mounted bone marrow aspirate). It is recommended that testes be fixed by immersion in Bouin's or modified Davidson's fixative (16) (17) (18); formalin fixation is not recommended for these tissues. The tunica albuginea may be gently and shallowly punctured at the both poles of the organ with a needle to permit rapid penetration of the fixative. The clinical and other findings may suggest the need to examine additional tissues. Also any organs considered likely to be target organs based on the known properties of the test chemical should be preserved.

67. The following tissues may give valuable indication for endocrine-related effects: Gonads (ovaries and testes), accessory sex organs (uterus including cervix, epididymides, seminal vesicles with coagulation glands, dorsolateral and ventral prostate), vagina, pituitary, male mammary gland and adrenal gland. Changes in male mammary glands have not been sufficiently documented but this parameter may be very sensitive to substances with estrogenic action. Observation of organs/tissues that are not listed in paragraph 66 is optional.

68. Dead pups and pups killed at day 13 post-partum, or shortly thereafter, should, at least, be carefully examined externally for gross abnormalities. Particular attention should be paid to the external reproductive genitals which should be examined for signs of altered development.

Histopathology

69. Full histopathology should be carried out on the preserved organs and tissues of the selected animals in the control and high dose groups (with special emphasis on stages of spermatogenesis in the male gonads and histopathology of interstitial testicular cell structure). The thyroid gland from pups and from the remaining adult animals may be examined when necessary. These examinations should be extended to animals of other dosage groups, if treatment-related changes are observed in the high dose group. The Guidance on histopathology (10) details extra information on dissection, fixation, sectioning and histopathology of endocrine tissues.

70. All gross lesions should be examined. To aid in the elucidation of NOAELs, target organs in other dose groups should be examined, particularly in groups claimed to show a NOAEL.

71. When a satellite group is used, histopathology should be performed on tissues and organs identified as showing effects in the treated groups.

Sample cryopreservation

72. Excess plasma as well as tissues or parts of tissues not preserved for histopathology may be preserved for additional investigations such as omics. Recommended procedures for preservation for omics are available in the OECD Guidance document No. 409 (19).

73. Care should be taken when considering additional sample preservation so that this does not compromise the standard parameters.

DATA AND REPORTING

Data

74. Individual animal data should be provided. Additionally, all data should be summarised in tabular form, showing for each test group the number of animals at the start of the test, the number of animals found dead during the test or euthanized for humane reasons, the time of any death or euthanasia, the number of fertile animals, 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 histopathological changes, and all relevant litter data. A tabular summary report format, which has proven to be very useful for the evaluation of reproductive/developmental effects, is given in Annex 3.

75. When possible, numerical results should be evaluated by an appropriate and general acceptable statistical method. Comparisons of the effect along a dose range should avoid the use of multiple t-tests. The statistical methods should be selected during the design of the study. Statistical analysis of AGD and nipple retention should be based on individual pup data, taking litter effects into account. Where appropriate, the litter is the unit of analysis. Statistical analysis of pup body weight should be based on individual pup data, taking litter size into account. Due to the limited dimensions of the study, statistical analyses in the form of tests for "significance" are of limited value for many endpoints, especially reproductive endpoints. Some of the most widely used methods, especially parametric tests for measures of central tendency, are inappropriate. If statistical analyses are used then the method chosen should be appropriate for the distribution of the variable examined and be selected prior to the start of the study.

Evaluation of results

76. The findings of this toxicity study should be evaluated in terms of the observed effects, necropsy and microscopic findings. The evaluation will include the relationship between the dose of the test chemical and the presence or absence, incidence and severity of abnormalities, including gross lesions, identified target organs, infertility, clinical abnormalities, affected reproductive and litter performance, body weight changes, effects on mortality and any other toxic effects.

77. Because of the short period of treatment of the male, the histopathology of the testes and epididymides should be considered along with the fertility data, when assessing male reproduction effects. The use of historic control data on reproduction/development (e.g. for litter size, AGD, nipple retention, serum T4 levels), where available, may also be useful as an aid to the interpretation of the study.

78. For quality control it is proposed that historical control data are collected and that for numerical data coefficients of variation are calculated, especially for the parameters linked with endocrine disrupter detection. These data can be used for comparison purposes when actual studies are evaluated.

Test report

79. The test report should include the following information:

Test chemical:

- source, lot number, limit date for use, if available
- stability of the test chemical, if known.

Mono-constituent substance:

- physical appearance, water solubility, and additional relevant physicochemical properties;
- chemical identification, such as IUPAC or CAS name, CAS number, SMILES or InChI code, structural formula, purity, chemical identity of impurities as appropriate and practically feasible, etc.

Multi-constituent substance, UVBCs and mixtures:

- characterised as far as possible by chemical identity (see above), quantitative occurrence and relevant physicochemical properties of the constituents.

Vehicle (if appropriate):

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

Test animals:

- species/strain used;
- number, age and sex of animals;
- source, housing conditions, diet, etc.;
- individual weights of animals at the start of the test.
- justification for species if not rat

Test conditions:

- rationale for dose level selection;
- details of test chemical formulation/diet preparation, achieved concentration, stability and homogeneity of the preparation;
- details of the administration of the test chemical;
- conversion from diet/drinking water test chemical concentration (ppm) to the actual dose (mg/kg body weight/day), if applicable;
- details of food and water quality;
- detailed description of the randomisation procedure to select pups for culling, if culled;
- report whether (yes/no) blinding was applied in the study;
- Samples cryopreserved (if applicable).

Results:

- body weight/body weight changes;
- food consumption and water consumption, if applicable;
- toxic response data by sex and dose, including fertility, gestation, and any other signs of toxicity;
- gestation length;
- toxic or other effects on reproduction, offspring, postnatal growth, etc.;
- nature, severity and duration of clinical observations (whether reversible or not);
- sensory activity, grip strength and motor activity assessments;
- haematological tests with relevant base-line values;
- clinical biochemistry tests with relevant base-line values;
- number of adult females with normal or abnormal oestrous cycle and cycle duration;
- number of live births and post implantation loss;
- number of pups with grossly visible abnormalities; gross evaluation of external genitalia, number of runts;
- time of death during the study or whether animals survived to termination;
- number of implantations, litter size and litter weights at the time of recording;
- pup body weight data
- AGD of all pups (and body weight on day of AGD measurement)
- nipple retention in male pups,
- thyroid hormone levels, day 13 pups and adult males (and dams and day 4 pups if measured)

- body weight at sacrifice and organ weight data for the parental animals;
- necropsy findings;
- a detailed description of histopathological findings;
- absorption data (if available);
- statistical treatment of results, where appropriate.

Discussion of results.

Conclusions.

Interpretation of Results

80. The study will provide evaluations of reproduction/developmental toxicity associated with administration of repeated doses. In particular, since emphasis is placed on both general toxicity and reproduction/developmental toxicity endpoints, the results of the study will allow for the discrimination between reproduction/developmental effects occurring in the absence of general toxicity and those which are only expressed at levels that are also toxic to parent animals (see paragraphs 7-11). It could provide an indication of the need to conduct further investigations and could provide guidance in the design of subsequent studies. OECD Guidance Document 43 should be consulted for aid in the interpretation of reproduction and developmental results (20). OECD Guidance Document 106 on Histologic Evaluation of Endocrine and Reproductive Tests in Rodents (16) provides information on the preparation and evaluation of (endocrine) organs and vaginal smears that may be helpful for this TG.

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ANNEX 1: DEFINITIONS (see also (21) OECD GD 150)

Androgenicity is the capability of a chemical to act like a natural androgenic hormone (e.g. testosterone) in a mammalian organism.

Antiandrogenicity is the capability of a chemical to suppress the action of a natural androgenic hormone (e.g. testosterone) in a mammalian organism.

Antioestrogenicity is the capability of a chemical to suppress the action of a natural oestrogenic hormone (e.g. oestradiol 17ß) in a mammalian organism.

Antithyroid activity is the capability of a chemical to suppress the action of a natural thyroid hormone (e.g. T3) in a mammalian organism.

Developmental toxicity: the manifestation of reproductive toxicity, representing pre-, peri- post-natal, structural, or functional disorders in the progeny.

Dose is the amount of test chemical administered. The dose is expressed as weight of test chemical per unit body weight of test animal per day (e.g. mg/kg body weight/day), or as a constant dietary concentration.

Dosage is a general term comprising dose, its frequency and the duration of dosing.

Evident toxicity is a general term describing clear signs of toxicity following administration of test chemical. These should be sufficient for hazard assessment and should be such that an increase in the dose administered can be expected to result in the development of severe toxic signs and probable mortality.

Impairment of fertility represents disorders of male or female reproductive functions or capacity.

Maternal toxicity: adverse effects on gravid females, occurring either specifically (direct effect) or not specifically (indirect effect) and being related to the gravid state.

NOAEL is the abbreviation for no-observed-adverse-effect level. This is the highest dose level where no adverse treatment-related findings are observed due to treatment.

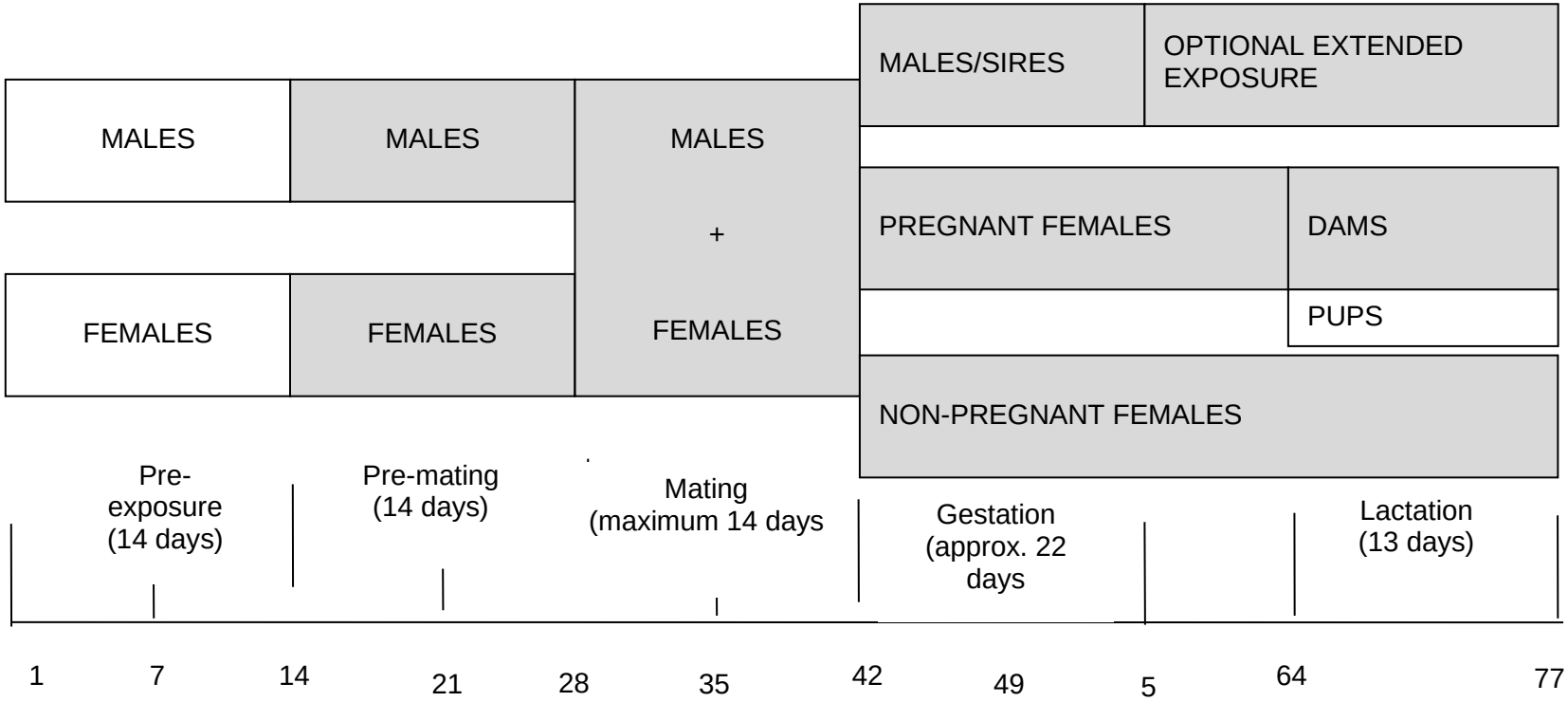
Oestrogenicity is the capability of a chemical to act like a natural oestrogenic hormone (e.g. oestradiol 17ß) in a mammalian organism.

Reproduction toxicity represents harmful effects on the progeny and/or an impairment of male and female reproductive functions or capacity.

Thyroid activity is the capability of a chemical to act like a natural thyroid hormone (e.g. T3) in a mammalian organism.

Validation is a scientific process designed to characterise the operational requirements and limitations of a test method and to demonstrate its reliability and relevance for a particular purpose.

ANNEX 2: DIAGRAM OF THE EXPERIMENTAL SCHEDULE, INDICATING THE MAXIMUM STUDY DURATION, BASED ON A FULL 14-DAY MATING PERIOD



Start of the study

Pre-exposure evaluation of oestrous cyclicity, followed by daily monitoring of vaginal smears from the beginning of treatment until evidence of mating

Dosing

Without dosing

Haematology/ clinical chemistry in males and females (optional)

Necropsy males/sires
Functional observations in males (optional)
Haematology/clinical chemistry in males, when killed (after dosing period of at least 4 weeks)

Parturition (PND 0) to PND 4:
AGD in all pups (PND 0-PND 4; same day)
Termination of 2 pups per litter for T4 (PND 4)

Day 13 post-partum
Necropsy females and pups
Necropsy males/sires (optional).
Functional observations in males (optional) and females
Haematology/clinical chemistry in males and females (optional)
Nipple retention in male pups.

ANNEX 3: TABULAR SUMMARY REPORT OF EFFECTS ON REPRODUCTION/ DEVELOPMENT

OBSERVATIONS	VALUES				
Dosage (units).....	**0 (control)**	**. . .**	**. . .**	**. . .**	**. . .**
Pairs started (N)					
Oestrus cycle (at least mean length and frequency of irregular cycles)					
Females showing evidence of copulation (N)					
Females achieving pregnancy (N)					
Conceiving days 1 - 5 (N)					
Conceiving days 6 - . . .[1] (N)					
Pregnancy≤21 days (N)					
Pregnancy = 22 days (N)					
Pregnancy ≥ 23 days (N)					
Dams with live young born (N)					
Dams with live young at day 4 pp (N)					
Implants/dam (mean)					
Live pups/dam at birth (mean)					
Live pups/dam at day 4 (mean)					
Sex ratio (m/f) at birth (mean)					
Sex ratio (m/f) at day 4 (mean)					
Litter weight at birth (mean)					
Litter weight at day 4 (mean)					
Pup weight at birth (mean)					
Pup weight at the time of AGD measurement(mean males, mean females)					
Pup AGD on the same postnatal day, birth- day 4 (mean males, mean females, note PND)					
Pup weight at day 4 (mean)					
Pup weight at day 13 (mean)					
Male pup nipple retention at day 13 (mean)					
ABNORMAL PUPS					
Dams with 0					
Dams with 1					
Dams with ≥ 2					
LOSS OF OFFSPRING					
Pre-natal (implantations minus live births)					
Females with 0					
Females with 1					
Females with 2					
Females with ≥ 3					
Post-natal (live births minus alive at post natal day 13)					
Females with 0					
Females with 1					

[1] last day of the mating period

Females with 2					
Females with ≥ 3					