OECD/OCDE 478
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OECD GUIDELINE FOR TESTING OF CHEMICALS

Rodent Dominant Lethal Test

INTRODUCTION

1. The OECD Guidelines for the Testing of Chemicals are periodically reviewed in the light of scientific progress, changing regulatory needs, and animal welfare considerations. The original Test Guideline 478 was adopted in 1984. This modified version of the Test Guideline reflects more than thirty years of experience with this test and the potential for integrating or combining this test with other toxicity tests such as developmental, reproductive toxicity, or genotoxicity studies; however due to its limitations and the use of a large number of animals this assay is not intended for use as a primary method, but rather as a supplemental test method which can only be used when there is no alternative for regulatory requirements. Combining toxicity testing has the potential to spare large numbers of animals from use in toxicity tests. A document that provides succinct information on genetic toxicology testing and an overview of the recent changes that were made to these Test Guidelines has been developed (1).

2. The purpose of the Dominant lethal (DL) test is to investigate whether chemicals produce mutations resulting from chromosomal aberrations in germ cells. In addition, the dominant lethal test is relevant to assessing genotoxicity because, although they may vary among species, factors of *in vivo* metabolism, pharmacokinetics and DNA-repair processes are active and contribute to the response. Induction of a DL mutation after exposure to a test chemical indicates that the chemical has affected germinal tissue of the test animal.

3. DL mutations cause embryonic or fetal death. Induction of DL mutation after exposure to a test chemical indicates that the chemical has affected the germ cells of the test animal.

4. A DL assay is useful for confirmation of positive results of tests using somatic *in vivo* endpoints, and is a relevant endpoint for the prediction of human hazard and risk of genetic diseases transmitted through the germline. However, this assay requires a large number of animals and is labour-intensive; as a result, it is very expensive and time-consuming to conduct. Because the spontaneous frequency of dominant lethal mutations is quite high, the sensitivity of the assay for detection of small increases in the frequency of mutations is generally limited.

5. Definitions of key terms are set out in Annex1.

INITIAL CONSIDERATIONS

6. The test is most often conducted in mice (2) (3) (4) but other species, such as rats (5) (6) (7) (8), may in some cases be appropriate if scientifically justified. DLs generally are the result of gross

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chromosomal aberrations (structural and numerical abnormalities) (9) (10) (11), but gene mutations cannot be excluded. A DL mutation is a mutation occurring in a germ cell per se, or is fixed post fertilization in the early embryo, that does not cause dysfunction of the gamete, but is lethal to the fertilized egg or developing embryo.

7. Individual males are mated sequentially to virgin females at appropriate intervals. The number of matings following treatment is dependent on the ultimate purpose of the DL study (Paragraph 23) and should ensure that all phases of male germ cell maturation are evaluated for DLs (12).

8. If there is evidence that the test chemical, or its metabolite(s), will not reach the testis, it is not appropriate to use this test.

PRINCIPLE OF THE TEST METHOD

9. Generally, male animals are exposed to a test chemical by an appropriate route of exposure and mated to untreated virgin females. Different germ cell types can be tested by the use of sequential mating intervals. Following mating, the females are euthanized after an appropriate period of time, and their uteri are examined to determine the numbers of implants and live and dead embryos. The dominant lethality of a test chemical is determined by comparing the live implants per female in the treated group with the live implants per female in the vehicle/solvent control group. The increase of dead implants per female in the treated group over the dead implants per female in the control group reflects the test-chemical-induced post-implantation loss. The post-implantation loss is calculated by determining the ratio of dead to total implants in the treated group compared to the ratio of dead to total implants in the control group. Pre-implantation loss can be estimated by comparing corpora lutea counts minus total implants or the total implants per female in treated and control groups.

VERIFICATION OF LABORATORY PROFICIENCY

10. Competence in this assay should be established by demonstrating the ability to reproduce dominant lethal frequencies from published data (e.g. (13) (14) (15) (16) (17) (18)) with positive control substances (including weak responses) such as those listed in Table 1, and vehicle controls and obtaining negative control frequencies that are consistent acceptable range of data (see references above) or with the laboratory's historical control distribution, if available.

DESCRIPTION OF THE METHOD

Preparations

Selection of animal species

11. Commonly used laboratory strains of healthy sexually mature animals should be employed. Mice are commonly used but rats may also be appropriate. Any other appropriate mammalian species may be used, if scientific justification is provided in the report.

Animal housing and feeding conditions

12. For rodents, the temperature in the animal room should be 22°C (±3°C). Although the relative humidity ideally should be 50-60%, it should be at least 40% and preferably not exceed 70%, other than during room cleaning. Lighting should be artificial, the sequence being 12 hours light, followed by 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 route. Prior to treatment or mating, rodents should be housed in small groups (no more than five) of the same sex if no aggressive behaviour is expected or observed, preferably in solid cages with appropriate environmental enrichment. Animals may be housed individually if scientifically justified.

Preparation of the animals

13. Healthy and sexually mature male and female adult animals are randomly assigned to the control and treatment groups. The individual animals are identified uniquely using a humane, minimally invasive method (e.g. by ringing, tagging, micro-chipping, or biometric identification, but not toe and ear clipping) and acclimated to the laboratory conditions for at least five days. Cages should be arranged in such a way that possible effects due to cage placement are minimized. Cross contamination by the positive control and the test chemical should be avoided. At the commencement of the study, the weight variation of animals should be minimal and not exceed ± 20% of the mean weight of each sex.

Preparation of doses

14. Solid test chemicals should be dissolved or suspended in appropriate solvents or vehicles or admixed in diet or drinking water prior to dosing of the animals. Liquid test chemicals may be dosed directly or diluted prior to dosing. For inhalation exposures, test materials can be administered as gas, vapour, or a solid/liquid aerosol, depending on their physicochemical properties. Fresh preparations of the test chemical should be employed unless stability data demonstrate the acceptability of storage and define the appropriate storage conditions.

Test Conditions

Solvent/vehicle

15. The solvent/vehicle should not produce toxic effects at the dose volumes used, and should not be suspected of chemical reaction with the test chemical. If other than well-known solvents/vehicles are used, their inclusion should be supported with reference data indicating their compatibility. It is recommended that wherever possible, the use of an aqueous solvent/vehicle should be considered first. Examples of commonly used compatible solvents/vehicles include water, physiological saline, methylcellulose solution, carboxymethyl cellulose sodium salt solution, olive oil and corn oil.

Positive controls

16. Concurrent positive control animals should always be used unless the laboratory has demonstrated proficiency in the conduct of the test and has used the test routinely in the recent past (e.g. within the last 5 years). However, it is not necessary to treat positive control animals by the same route as animals receiving the test chemical, or sample all the mating intervals. The positive control substances should be known to produce DLs under the conditions used for the test. Except for the treatment, animals in the control groups should be handled in an identical manner to animals in the treated groups.

17. The doses of the positive control substances should be selected so as to produce weak or moderate effects that critically assess the performance and sensitivity of the assay, but which consistently produce positive dominant lethal effects. Examples of positive control substances, and appropriate doses, are included in Table 1.

Table 1. Examples of Positive Control Substances.

Substance [CAS no.] (reference no.)	Effective Dose range (mg/kg) (rodent species)	Administration Time (days)
Triethylenemelamine [51-18-3] (15)	0.25 (mice)	1
Cyclophosphamide [50-18-0] (19)	50-150 (mice)	5
Cyclophosphamide [50-18-0] (5)	25-100 (rats)	1
Ethyl methanesulphonate [62-50-0] (13)	100-300 (mice)	5
Monomeric Acrylamide [79-06-1] (17)	50 (mice)	5
Chlorambucil [305-03-3] (14)	25 (mice)	1

Negative controls

18. Negative control animals, treated with solvent or vehicle alone, and otherwise treated in the same way as the treatment groups, should be included for every sampling time (20). In the absence of historical or published control data showing that no DLs or other deleterious effects are induced by the chosen solvent/vehicle, untreated control animals should also be included for every sampling time in order to establish acceptability of the vehicle control.

PROCEDURE

Number of Animals

19. Individual males are mated sequentially at appropriate predetermined intervals (e.g. weekly intervals, Paragraphs 21 & 23) preferably to one virgin female. The number of males per group should be predetermined to be sufficient (in combination with the number of mated females at each mating interval) to provide the statistical power necessary to detect at least a doubling in DL frequency (Paragraph 44).

20. The number of females per mating interval should also be predetermined by statistical power calculations to permit the detection of at least a doubling in the DL frequency (i.e. sufficient pregnant females to provide at least 400 total implants) (20) (21) (22) (23) and that at least one dead implant per analysis unit (i.e. mating group per dose) is expected (24).

Administration Period and Mating Intervals

21. The number of mating intervals following treatment is governed by the treatment schedule and should ensure that all phases of male germ cell maturation are evaluated for DL induction (12) (25). For a single treatment up to five daily dose administrations, there should be 8 (mouse) or 10 (rat) matings

conducted at weekly intervals following the last treatment. For multiple dose administrations, the number of mating intervals may be reduced in proportion to the increased time of the administration period, but maintaining the goal of evaluating all phases of spermatogenesis (e.g. after a 28-day exposure, only 4 weekly matings are sufficient to evaluate all phased of spermatogenesis in the mouse). All treatment and mating schedules should be scientifically justified.

22. Females should remain with the males for at least the duration of one oestrus cycle (e.g. one week covers one oestrus cycle in both mice and rats). Females that did not mate during a one-week interval can be used for a subsequent mating interval. Alternatively, until mating has occurred, as determined by the presence of sperm in the vagina or by the presence of a vaginal plug.

23. The exposure and mating regimen used is dependent on the ultimate purpose of the DL study. If the goal is to determine whether a given chemical induces DL mutations *per se*, then the accepted method would be to expose an entire round of spermatogenesis (e.g. 7 weeks in the mouse, 5-7 treatments per week) and mate once at the end. However, if the goal is to identify the sensitive germ cell type for DL induction, then a single or 5 day exposure followed by weekly mating is preferred.

Dose Levels

24. If a preliminary range-finding study is performed because there are no suitable data already available to aid in dose selection, it should be performed in the same laboratory, using the same species, strain, sex, and treatment regimen to be used in the main study (26). The study should aim to identify the maximum tolerated dose (MTD), defined as the highest dose that will be tolerated without evidence of study-limiting toxicity, relative to the duration of the study period (for example, abnormal behaviour or reactions, minor body weight depression or hematopoietic system cytotoxicity), but not death or evidence of pain, suffering or distress necessitating humane euthanasia (27).

25. The MTD must also not adversely affect mating success (21).

26. Test chemicals with specific biological activities at low non-toxic doses (such as hormones and mitogens), and chemicals which exhibit saturation of toxicokinetic properties may be exceptions to the dose-setting criteria and should be evaluated on a case-by-case basis.

27. In order to obtain dose response information, a complete study should include a negative control group and a minimum of three dose levels generally separated by a factor of 2, but not greater than 4. If the test chemical does not produce toxicity in a range-finding study, or based on existing data, the highest dose for a single administration should be 2000 mg/kg body weight. However, if the test chemical does cause toxicity, the MTD should be the highest dose administered and the dose levels used should preferable cover a range from the maximum to a dose producing little or no toxicity. For not-toxic substances, the limit dose for an administration period of 14 days or more is 1000 mg/kg body weight/day, and for administration periods of less than 14 days the limit dose is 2000 mg/kg body weight/day.

Administration of Doses

28. The anticipated route of human exposure should be considered when designing an assay. Therefore, routes of exposures such as dietary, drinking water, subcutaneous, intravenous, topical, inhalation, oral (by gavage), or implantation may be chosen as justified. In any case, the route should be chosen to ensure adequate exposure of the target tissue(s). Intraperitoneal injection is not normally recommended since it is not an intended route of human exposure, and should only be used with specific

scientific justification. If the test chemical is admixed in diet or drinking water, especially in case of single dosing, care should be taken that the delay between food and water consumption and mating should be sufficient to allow detection of the effects (paragraph 31). The maximum volume of liquid that can be administered by gavage or injection at one time depends on the size of the test animal. The volume should not normally exceed 1 mL/100g body weight except in the case of aqueous solutions where a maximum of 2 mL/100g may be used. The use of volumes greater than this (if permitted by animal welfare legislation) should be justified. Variability in test volume should be minimized by adjusting the concentration to ensure a constant volume in relation to body weight at all dose levels.

Observations

29. General clinical observations of the test animals should be made and clinical signs recorded at least once a day, preferably at the same time(s) each day and considering the peak period of anticipated effects after dosing. At least twice daily during the dosing period, all animals should be observed for morbidity and mortality. All animals should be weighed at the beginning of the study and at least once a week during repeated dose studies, and at the time of euthanasia. Measurements of food consumption should be made at least weekly. If the test chemical is administered via the drinking water, water consumption should be measured at each change of water and at least weekly. Animals exhibiting non-lethal indicators of excess toxicity should be euthanised prior to completion of the test period (27).

Tissue Collection and Processing

30. Females are euthanised in the second half of pregnancy at gestation day (GD) 13 for mice and GD 14-15 for rats. Uteri are examined for dominant lethal effects to determine the number of implants, live and dead embryos, and corpora lutea.

31. The uterine horns and ovaries are exposed for counting of corpora lutea, and fetuses are removed, counted, and weighted. Care should be taken to examine the uteri for resorptions obscured by live fetuses and to ensure that all resorptions are enumerated. Fetal mortality is recorded. The number of successfully impregnated females and the number of total implantations, pre-implantation losses, and post-implantation mortality (included early and late resorptions) also are recorded. In addition, the visible fetuses may be preserved in Bouin's fixative for at least 2 weeks followed by examination for major external malformations (28) to provide additional information on the reproductive and developmental effects of the test agent.

DATA AND REPORTING

Treatment of Results

32. Data should be tabulated to show the number of males mated, the number of pregnant females, and the number of non-pregnant females. Results of each mating, including the identity of each male and female, should be reported individually. The mating interval, dose level for treated males, and the numbers of live implants and dead implants should be enumerated for each female.

33. The post-implantation loss is calculated by determining the ratio of dead to total implants from the treated group compared to the ratio of dead to total implants from the vehicle/solvent control group.

34. Pre-implantation loss is calculated as the difference between the number of corpora lutea and the number of implants, or as a reduction in the average number of implants per female in comparison with control matings. Where pre-implantation loss is estimated, it should be reported.

35. The Dominant Lethal factor is estimated as: (post-implantation deaths/total implantations per female) x 100.

36. Data on toxicity and clinical signs (as per Paragraph 29) should be reported.

Acceptability Criteria

37. The following criteria determine the acceptability of a test.

a) Concurrent negative control is consistent with published norms for historical negative control data, and the laboratory's historical control data if available (see Paragraphs 10 and 18).

b) Concurrent positive controls induce responses that are consistent with published norms for historic positive control data, or the laboratory's historical positive control database, if available, and produce a statistically significant increase compared with the negative control (see Paragraphs 17 and 18).

c) Adequate number total implants and doses have been analysed (Paragraph 20).

d) The criteria for the selection of top dose are consistent with those described in Paragraphs 24 and 27.

Evaluation and Interpretation of Results

38. At least three treated dose groups should be analysed in order to provide sufficient data for dose-response analysis.

39. Providing that all acceptability criteria are fulfilled, a test chemical is considered a clear positive if:

a) at least one of the test doses exhibits a statistically significant increase compared with the concurrent negative control;

b) the increase is dose-related in at least one experimental condition (e.g. a weekly mating interval) when evaluated with an appropriate test; and,

c) any of the results are outside of the acceptable range of negative control data, or the distribution of the laboratory's historical negative control data (e.g. Poisson-based 95% control limit) if available.

The test chemical is then considered able to induce dominant lethal mutations in germ cells of the test animals. Recommendations for the most appropriate statistical methods are described in Paragraph 44; other recommend statistical approaches can also be found in the literature (20) (21) (22) (24) (29). Statistical tests used should consider the animal as the experimental unit.

40. Providing that all acceptability criteria are fulfilled, a test chemical is considered a clear negative if:

a) none of the test doses exhibits a statistically significant increase compared with the concurrent negative control;
b) there is no dose-related increase in any experimental condition; and

c) all results are within acceptable range of negative control data, or the laboratory's historical negative control data (e.g. Poisson-based 95% control limit), if available.

The test chemical is then considered unable to induce dominant lethal mutations in germ cells of the test animals.

41. There is no requirement for verification of a clear positive or a clear negative response.

42. If the response is not clearly negative or positive, and in order to assist in establishing the biological relevance of a result (e.g. a weak or borderline increase), the data should be evaluated by expert judgment and/or further investigations using the existing experimental data, such as consideration whether the positive result is outside the acceptable range of negative control data, or the laboratory's historical, negative control data (30).

43. In rare cases, even after further investigations, the data set will preclude making a conclusion of positive or negative results, and will therefore be concluded as equivocal.

44. Statistical tests used should consider the male animal as the experimental unit. While it is possible that count data (e.g. number of implants per female) may be Poisson distributed and/or proportions (e.g. proportion of dead implants) may be binomially distributed, it is often the case that such data are overdispersed (31). Accordingly, statistical analysis should first employ a test for over-underdispersion using variance tests such as Cochran's binomial variance test (32) or Tarone's C(α) test for binomial overdispersion (31) (33). If no departure from binomial dispersion is detected, trends in proportions across dose levels may be tested using the Cochran-Armitage trend test (34) and pairwise comparisons with the control group may be tested using Fisher's exact test (35). Likewise, if no departure from Poisson dispersion is detected, trends in counts may be tested using Poisson regression (36) and pairwise comparisons with the control group may be tested within the context of the Poisson model, using pairwise contrasts (36). If significant overdispersion or underdispersion is detected, nonparametric methods are recommended (23) (31). These include rank-based tests, such as the Jonckheere-Terpstra test for trend (37) and Mann-Whitney tests (38) for pairwise comparisons with the vehicle/solvent control group, as well as permutation, resampling, or bootstrap tests for trend and pairwise comparisons with the control group (31) (39).

45. A positive DL assay provides evidence for the genotoxicity of the test chemical in the germ cells of the treated male of the test species.

46. Consideration of whether the observed values are within or outside of the historical control range can provide guidance when evaluating the biological significance of the response (40).

Test Report

47. The test report should include the following information.

Summary.

Test chemical:

- source, lot number, limit date for use, if available;
- stability of the test chemical itself, if known;
- solubility and stability of the test chemical in solvent, if known;
- measurement of pH, osmolality, and precipitate in the culture medium to which the test chemical was added, as appropriate.

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

Test chemical preparation:

- justification for choice of vehicle;
- solubility and stability of the test chemical in the solvent/vehicle, if known;
- preparation of dietary, drinking water or inhalation formulations;
- analytical determinations on formulations (e.g. stability, homogeneity, nominal concentrations) when conducted.

Test animals:

- species/strain used and justification for the choice;
- number, age and sex of animals;
- source, housing conditions, diet, etc.;
- method of uniquely identifying the animals;
- for short-term studies: individual body weight of the male animals at the start and end of the test; for studies longer than one week: individual body weights during the study and food consumption. Body weight range, mean and standard deviation for each group should be included.

Test conditions:

- positive and negative (vehicle/solvent) control data;
- data from the range-finding study;
- rationale for dose level selection;
- details of test chemical preparation;
- details of the administration of the test chemical;
- rationale for route of administration;
- methods for measurement of animal toxicity, including, where available, histopathological or hematological analyses and the frequency with which animal observations and body weights were taken;
- methods for verifying that the test chemical reached the target tissue, or general circulation, if negative results are obtained;
- actual dose (mg/kg body weight/day) calculated from diet/drinking water test chemical concentration (ppm) and consumption, if applicable;
- details of food and water quality;
- details on cage environment enrichment;
- detailed description of treatment and sampling schedules and justifications for the choices;

- method of analgesia
- method of euthanasia;
- procedures for isolating and preserving tissues;
- source and lot numbers of all kits and reagents (where applicable);
- methods for enumeration of DLs;
- mating schedule;
- methods used to determine that mating has occurred;
- time of euthanasia
- criteria for scoring DL effects, including, corpora lutea, implantations, resorptions and pre-implantation losses, live implants, dead implants.

Results:

- animal condition prior to and throughout the test period, including signs of toxicity;
- male body weight during the treatment and mating periods;
- number of mated females;
- dose-response relationship, where possible;
- concurrent and historical negative control data with ranges, means and standard deviations;
- concurrent positive control data;
- tabulated data or each dam including: number of corpora lutea per dam; number of implantations per dam; number of resorptions and pre-implantation losses per dam; number of live implants per dam; number of dead implants per dam; fetus weights;
- the above data summarized for each mating period and dose, with Dominant Lethal frequencies;
- statistical analyses and methods applied.

Discussion of the results.

Conclusion.

LITERATURE

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ANNEX 1

DEFINITIONS

Corpora luteum (lutea): the hormonal secreting structure formed on the overy at the site of a follicle that has released the egg. The number of corpora lutea in the ovaries corresponds to the number of eggs that were ovulated.

Dominant Lethal Mutation: a mutation occurring in a germ cell, or is fixed after fertilization, that causes embryonic or foetal death.

Fertility rate: the number of mated pregnant female over the number of mated females.

Mating interval: the time between the end of exposure and mating of treated males. By controlling this interval, chemical effects on different germ cell types can be assessed. In the mouse mating during the 1, 2, 3, 4, 5, 6, 7 and 8 week after the end of exposure measures effects in sperm, condensed spermatids, round spermatids, pachytene spermatocytes, early spermatocytes, differentiated spermatogonia, differentiating spermatogonia and stem cell spermatogonia.

Preimplantation loss: the difference between the number of implants and the number of corpora lutea. It can also be estimated by comparing the total implants per female in treated and control groups.

Postimplantation loss: the ratio of dead implant in the treated group compared to the ratio of dead to total implants in the control group.

ANNEX 2

TIMIING OF SPERMATOGENESIS IN MAMMALS

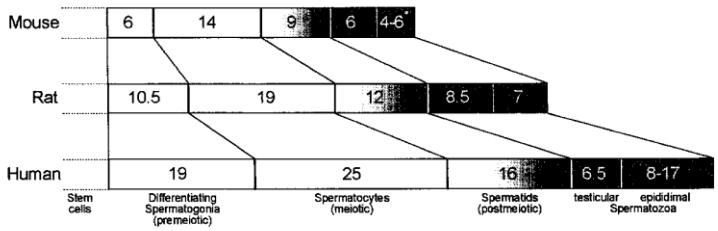

Fig. 1. Comparison of the duration (days) of male germ cell development in mice, rats and humans. DNA repair does not occur during the periods indicated by shading.

A schematic of spermatogenesis in the mouse, rat and human is shown above (taken from Adler, 1996). Undifferentiated spermatogonia include: A-single; A-paired; and A-aligned spermatogonia (Hess and de Franca, 2008). A-single is considered the true stem cells; therefore, to assess effects on stem cells at least 49 days (in the mouse) must pass between the last injection of the test chemical and mating.

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