

OECD GUIDELINE FOR TESTING OF CHEMICALS

Draft Rodent Dominant Lethal Test 478

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. Modifications within the current version reflect nearly thirty years of experience with this test.
2. Dominant lethal (DL) mutations cause embryonic or foetal death. Induction of a DL mutation after exposure to a test chemical indicates that the chemical has affected germinal tissue of the test animal
3. This rodent *in vivo* test is relevant to assessing mutagenic effects (mainly chromosome damage) in male germ cells. Such responses are dependent upon *in vivo* metabolism, pharmacokinetics, DNA repair processes, and translesion DNA synthesis, although these may vary among species, and among different types of DNA damage.
4. A DL assay is useful for confirmation of 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.
5. Definitions of key terms are set out in the Annex.

INITIAL CONSIDERATIONS

6. DLs generally are the result of chromosomal aberrations (structural and numerical anomalies), but gene mutations and toxicity 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. This *in vivo* test is designed to investigate whether chemical and physical agents produce mutations resulting from chromosomal aberrations in germ cells. In addition, the dominant lethal test is relevant to assessing genetic hazard in that it incorporates *in vivo* metabolism, pharmacokinetics and DNA-repair processes into the assessment.
8. If there is evidence that the test chemical, or a reactive metabolite, will not reach male germ cells (i.e. spermatogonia stem cells and/or spermatogonia), it is not appropriate to use this test

9. The exposure and mating regimen used is dependent on the ultimate purpose of the DL study. If the goal is to identify the sensitive stage for DL induction, then a single or 5 day exposure followed by weekly mating intervals is preferred. However, 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 and mate once at the end.

PRINCIPLE OF THE TEST METHOD

10. Generally, male animals are exposed to a test chemical 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 agent 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-agent-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 from corpora lutea counts or by comparing the total implants per female in treated and control groups.

VERIFICATION OF LABORATORY PROFICIENCY

11. Competence in this assay should be established by demonstrating the ability to reproduce dominant lethal frequencies from published data (e.g. 14, 18, 21, 28) with positive control chemicals (including weak responses) such as those listed in Table 1, and vehicle controls. Prior demonstration of proficiency is not necessary for laboratories that routinely perform developmental and reproductive toxicity tests which involve the examination of uterine contents.

DESCRIPTION OF THE METHOD (See references 1, 2, 3, 4, 5, 7, 8, 13, 20) for background)

12. Several treatment schedules can be used. The most widely used requires single administration of the test chemical. Other treatment schedules, such as treatment on five consecutive days or more, may be used if justified by the investigator.

13. Individual males are mated sequentially to virgin females at appropriate intervals. The number of matings following treatment is governed by the treatment schedule and should ensure that all phases of male germ cell maturation are evaluated for DLs. Females are euthanised during the second half of pregnancy, and their uteri are examined to determine the total number of implants and the number of live and dead embryos.

Preparations

Selection of animal species

14. Mice or rats may be used. The choice of species should be scientifically justified (e.g., the need to correlate, or combine, with other toxicity studies, or metabolism known to be more representative of human metabolism).

Animal housing and feeding conditions

15. The temperature in the experimental animal room ideally should be 22°C ($\pm 3^\circ\text{C}$). Although the relative humidity ideally should be 50-60%, it should be at least 30% and preferably not exceed 70%, other than during room cleaning. Lighting should be artificial, with a daily sequence of 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, males and females should be housed apart in small groups (no more than five), unless aggressive behavior is expected or observed.

Preparation of the animals

16. Healthy young male and female adult animals (8-12 weeks old at start of treatment) are randomly assigned to the control and treatment groups. The animals are identified uniquely 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. At the commencement of the study, the weight variation of animals should be minimal and not exceed $\pm 20\%$ of the mean weight of each sex.

Preparation of doses

17. 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.

Test Conditions

Solvent/vehicle

18. 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.

Positive controls

19. Concurrent positive control animals should always be used; however, it is not necessary to treat them by the same route as the test chemical, or sample all the mating intervals. The positive control chemicals should be known to produce DLs under the conditions used for the test. Except for treatment with the test chemical, animals in the control groups should be handled in an identical manner to animals in the treated groups.

20. The doses of the positive control chemicals 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 chemicals, and appropriate doses, are included in Table 1.

Table 1. Examples of Positive Control Chemicals.

Chemical [CAS no.] (reference no.)	Effective Dose range (mg/kg)(rodent species)	Administration Time (days)
triethylenemelamine [51-18-3] (17)	0.25 (mice)	1
cyclophosphamide [50-18-0] (20)	50-150 (mice)	5
ethyl methanesulphonate [62-50-0] (14)	100-300 (mice)	5
bleomycin [9041-93-4] (27)	50-150 (mice)	5

Negative controls

21. Negative controls, treated with solvent or vehicle alone, and otherwise treated in the same way as the treatment groups, should be included for every sampling time. 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 controls should also be included for every sampling time in order to establish acceptability of the vehicle control.

PROCEDURE

Number of Animals

22. Individual males are mated sequentially at appropriate predetermined intervals (Paragraph 25) preferably to one virgin female (although two females per male can be used, if necessary). 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 48).

23. For optimum results, males may be mated once before treatment in order to “flush” the reproductive track of sperm produced prior to exposure of the test chemical.

24. The number of females per mating interval also should be predetermined by statistical power calculations to permit the detection of at least a doubling in the DL frequency (e.g. about 40-50 pregnant females per mating interval sufficient to provide at least 400 total implants (1), and that each analysis unit contains sufficient numbers of females and implants so that at least one dead implant is expected (30). .

Administration Period and Mating Intervals

25. 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. For a single treatment up to five daily dose administrations, there should be 8 matings conducted at weekly intervals for the mouse, and 10 weekly matings for the rat. 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. All treatment and mating schedules should be scientifically justified.

26. For optimum results, males may be mated once before treatment in order to “flush” the reproductive track of sperm produced prior to exposure of the test chemical.

27. Females should remain with the males for at least the duration of one oestrus cycle (e.g. one week), or alternatively until mating has occurred as determined by the presence of sperm in the vagina or by the presence of a vaginal plug

28. 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 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

29. 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 (Fielder et al 8), according to current recommendations for conducting dose range-finding studies. The range-finding study should be based on the same dosing

regimen as the main test, and aim to identify the maximum tolerated dose (MTD), defined as the dose inducing slight toxic effects (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.

29. The MTD must also not adversely affect litter size

30. 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.

31. In the main study, in order to obtain dose response information, a complete study should include a negative vehicle/solvent control group (Paragraph 21) and a minimum of three, appropriately spaced dose levels. If the test chemical does produce toxicity in a range-finding study, or based on existing data, the MTD and two lower dose levels generally separated by a factor of 2, but by no greater than 4, should be selected. If the test chemical does produce toxicity, the limit dose plus two lower doses (as described above) should be selected. 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

30. In general, the anticipated route of human exposure should be considered when designing an assay. Usually, the test chemical is administered by gavage using a stomach tube or a suitable intubation cannula. Other routes of exposure (such as, drinking water, subcutaneous, intravenous, topical, inhalation, intratracheal, dietary, or implantation) may be acceptable when it leads to an exposure relevant to the expected route of human exposure. 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 sampling should be sufficient. Intraperitoneal injection is not normally recommended unless scientifically justified since it is not usually a physiologically relevant route of human exposure. 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 exceed 2 mL/100g body weight. The use of volumes greater than this should be justified. Except for irritating or corrosive chemicals, which will normally reveal exacerbated effects at higher concentrations, variability in test volume should be minimized by adjusting the concentration to ensure a constant volume at all dose levels.

Observations

33. General clinical observations of the test animals 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 should be observed for morbidity and mortality. All animals should be weighed at least once a week, and at 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

34. 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.

35. 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 subjected to external malformation examinations and preserved in Bouin's fixative for at least 2 weeks followed by sectioning and examination for major abnormalities (6).

DATA AND REPORTING

Treatment of Results

36. 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.

37. 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.

38. 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.

39. The Dominant Lethal factor is estimated as: $(\text{post-implantation deaths}/\text{total implantations per female}) \times 100$.

40. Data on toxicity and clinical signs (as per Paragraph 34) should be reported.

Acceptability Criteria

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

- a) Concurrent negative control is consistent with published norms for historical negative control data, or the laboratory's historical control data (see Paragraph 11).
- b) Concurrent positive controls fulfil the positivity criteria (see Paragraphs 19, 20).
- c) Adequate number total implants and doses have been analyzed (Paragraph 24).
- d) The criteria for the selection of top dose are consistent with those described in Paragraphs 21 and 22.

Evaluation and Interpretation of Results

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

43. Providing that all acceptability criteria are fulfilled, a test chemical is considered a clear positive if at least one of the test doses exhibits a statistically significant increase compared with the concurrent negative control, and the increase is dose-related in at least one experimental condition (e.g. a weekly mating interval). The test chemical is then considered able to induce dominant lethal mutations in spermatogonial cells of the test animals. Recommendations for the most appropriate statistical methods are described in Paragraph 48; other recommend statistical approaches can also be found in the literature (1, 23, 30). Statistical tests used should consider the animal as the experimental unit.

44. Providing that all acceptability criteria are fulfilled, a test chemical is considered a clear negative if none of the test doses exhibits a statistically significant increase compared with the concurrent negative control, and there is no dose-related increase in any experimental condition. The test chemical is then considered unable to induce dominant lethal mutations in germ cells of the test animals. In the case of a negative result, the likelihood that the test chemical, or its metabolites, reaches the target tissue should be discussed.

45. 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 distribution of the published, or the laboratory's historical, negative control data (19).

46. 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.

47. Statistical tests used should consider the male animal as the experimental unit. While it is possible that count data (e.g. number of 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 (24). Accordingly, statistical analysis should first employ a test for over- underdispersion using variance tests such as Cochran's binomial variance test (9) or Tarone's $C(\alpha)$ test for binomial overdispersion (27). If no departure from binomial dispersion is detected,

trends in proportions across dose levels may be tested using the Cochran-Armitage trend test (25) and pairwise comparisons with the control group may be tested using Fisher's exact test (11). Likewise, if no departure from Poisson dispersion is detected, trends in counts may be tested using Poisson regression (26) and pairwise comparisons with the control group may be tested within the context of the Poisson model, using pairwise contrasts (26). If significant overdispersion or underdispersion is detected, nonparametric methods are recommended (17, 24). These include rank-based tests, such as the Jonckheere-Terpstra test for trend (22) and Mann-Whitney tests (10) 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 (12, 24).

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

49. A test chemical for which the results do not meet the above criteria is considered negative in this assay. Germ cell exposure must be demonstrated to conclude that a test agent is negative in the assay.

50. 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 (16).

Test Report

51. The test report should include the following information.

Summary.

Test chemical:

- identification data and CAS n^o, if known;
- source, lot number if available;
- physical nature and purity;
- physicochemical properties relevant to the conduct of the study;
- stability of the test chemical, if known.

Solvent/vehicle:

- 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, if relevant;
- analytical determinations on formulations (e.g., stability, homogeneity, nominal concentrations).

Test animals:

- species/strain used and justification for the choice;
- number, age and sex of animals;
- source, housing conditions, diet, etc.;
- individual weight of the animals at the start of the test, including body weight range, mean and standard deviation for each group.

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 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 (where applicable);
- 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;
- body weight during the treatment and manifestation periods; body and organ weights at sacrifice;
- 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 on 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
- analytical determinations, if available
- statistical analyses and methods applied.

Discussion of the results.

Conclusion.

LITERATURE

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Definitions (to be added)