OECD GUIDELINE FOR THE TESTING OF CHEMICALS

Draft Mammalian Spermatogonial Chromosomal Aberration 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 483 was adopted in 1997. Modifications within the current version reflect nearly thirty years of experience with this test.

2. The purpose of the *in vivo* mammalian spermatogonial chromosomal aberration test is to identify those chemicals that cause structural aberrations in mammalian spermatogonial cells. Structural aberrations may be of two types, chromosome and chromatid. With the majority of chemical mutagens, induced aberrations are of the chromatid type; but chromosome-type aberrations also occur. This guideline is not designed to measure numerical abnormalities; the assay is not routinely used for this purpose.

3. This test measures chromosomal aberrations in spermatogonial germ cells and is, therefore, expected to be predictive of induction of heritable mutations in these germ cells.

4. Definitions used are set out in the Annex.

INITIAL CONSIDERATIONS

5. Rodents are routinely used in this test. This *in vivo* cytogenetic test detects chromosome aberrations in spermatogonial mitoses. Other target cells are not the subject of this guideline.

6. To detect chromatid-type aberrations in spermatogonial cells, the first mitotic cell division following treatment should be examined before these aberrations are lost in subsequent cell divisions. Additional information from treated spermatocytes can be obtained by meiotic chromosome analysis for chromosomal aberrations at diakinesis-metaphase I and metaphase II.

7. This *in vivo* test is designed to investigate whether chemical and physical agents produce chromosomal aberrations in germ cells. In addition, the spermatogonial test is relevant to assessing genetic hazard in that it incorporates *in vivo* metabolism, pharmacokinetics and DNA-repair processes into the assessment.

8. A number of generations of spermatogonia are present in the testis, and these different germ cell stages may have a spectrum of sensitivity to chemical treatment. Thus, the aberrations detected represent an aggregate response of treated spermatogonial cell populations, with the more numerous differentiated spermatogonial cells predominating.

9. If there is evidence that the test chemical, or a reactive metabolite, will not reach male germ cells (i.e. spematogonia stem cells and/or spematogonia), it is not appropriate to use this test.

PRINCIPLE OF THE TEST

10. Animals are exposed to the test chemical by an appropriate route of exposure and are euthanized at appropriate times after treatment. Prior to euthanasia, animals are treated with a metaphase-arresting agent (e.g., colchicine or Colcemid®). Chromosome preparations are then made from germ cells and stained, and metaphase cells are analyzed for chromosome aberrations.

VERIFICATION OF LABORATORY PROFICIENCY

11. Competency in this assay should be established by demonstrating the ability to reproduce expected results from published data for chromosomal aberration frequencies in spermatogonia with positive control chemicals (including weak responses) such as those listed in Table 1, and for vehicle controls (e.g. 1, 2, 3, 4, 5, 6, 12, 14).

DESCRIPTION OF THE METHOD (See references 1, 2, 3, 4, 7, 12 for background)

Preparations

Selection of animal species

12. Commonly used laboratory strains of healthy young adult animals should be employed. Male mice are commonly used; however, males of other appropriate mammalian species may be used. The scientific justification for using species other than mice (e.g. rats) should be provided in the report.

Animal Housing and feeding conditions

13. 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 30% and preferably not exceed 70% other than during room cleaning. Lighting should be artificial, the sequence being 12 hours light, 12 hours dark. For feeding, conventional laboratory diets may be used with an unlimited supply of drinking water. The choice of diet may be influenced by the need to ensure a suitable admixture of a test chemical when administered by this route. Rodents should be housed in small groups (no more than five per cage) of the same sex if no aggressive behaviour is expected, preferably in solid floor cages with appropriate environmental enrichment. Animals may be housed individually only if scientifically justified.

Preparation of the animals

14. Healthy young adult male animals (8-12 weeks old at start of treatment) are normally used, and are randomly assigned to the control and treatment groups. The individual 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 variation between individual animal weights and the mean weight of each corresponding sex group should be minimal and not exceed ± 20%.

Preparation of doses

15. 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

16. The solvent/vehicle should not produce toxic effects at the dose levels used and should not be capable 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

17. 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 time intervals. The positive control chemicals should be known to produce structural chromosomal aberrations *in vivo* in spermatogonial cells 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.

18. The doses of the positive control chemicals should be selected so as to produce weak to moderate effects that critically assess the performance and sensitivity of the assay, but which consistently produce positive frequencies of chromosomal aberrations in spermatogonia, and do not immediately reveal the identity of the coded slides to the reader. Examples of positive control chemicals are included in Table 1.

Table 1. Examples of positive control chemicals.

Chemical [CAS No.] (reference no.)
Cyclophosphamide (monohydrate) [CAS no. 50-18-0 (CAS no. 6055-19-2)] (12)
Cyclohexylamine [CAS no. 108-91-8] (4)
Mitomycin C [CAS no. 50-07-7] (1)

Monomeric acrylamide [CAS 79-06-1] (14)
Triethylenemelamine [CAS 51-18-3] (6)

Negative controls

19. 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 chromosomal aberrations or other deleterious effects are induced by the chosen solvent/vehicle, untreated controls also should be included for every sampling time in order to establish acceptability of the vehicle control.

PROCEDURE

Number of animals

20. Group sizes at study initiation should be established with the aim of providing minimum of 5 male animals. This number of animals per group is considered to be sufficient to provide adequate statistical power (i.e. generally able to detect at least a doubling in chromosomal aberration frequency when the negative control level is 1.0% or greater with 80% probability at a significance level of 0.05) (4). However, if the statistical power is insufficient, the number of animals should be increased as required.

Treatment schedule

21. Test chemicals are usually administered once (i.e. as a single treatment); other dose regimens may be used, provided they are scientifically justified.

22. In the highest dose group two sampling times after treatment are used. Since cell cycle kinetics can be influenced by the test chemical, one early and one late sampling time approximately 24 and 48 hours after treatment are used. For doses other than the highest dose, an early sampling time of 24 hours (i.e. the time necessary to complete 1.5 cell cycles) after treatment should be taken, unless another sampling time is known to be more appropriate and justified.

23. In addition, other sampling times may be used. For example in the case of chemicals that exert S-independent effects, earlier sampling times may be appropriate.

24. A repeat dose treatment regimen can be used, such as in conjunction with a test on another endpoint that uses a 28 day administration period (e.g. TG 488); however, additional animal groups would be required to accommodate different sampling times. Accordingly, the appropriateness of such a schedule needs to be justified scientifically on a case-by-case basis.

25. Prior to euthanasia, animals are injected intraperitoneally with an appropriate dose of a metaphase arresting chemical (e.g. Colcemid® or colchicine). Animals are sampled at an appropriate interval thereafter. For mice this interval is approximately 3 - 5 hours, for Chinese hamsters this interval is approximately 4 - 5 hours.

Dose levels

26. 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 (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 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. The highest dose may also be defined as a dose that produces some indication of toxicity in the spermatogonial cells (e.g. a reduction in the ratio of spermatogonial mitoses to first and second meiotic metaphases; this reduction should not exceed 50%).

28. 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.

29. In the main study, in order to obtain dose response information, a complete study should include a negative vehicle/solvent control group (Paragraph 19) 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

31. 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 should be observed for morbidity and mortality. In experiments using a prolonged treatment regimen, 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 euthanized prior to completion of the test period (11).

Chromosome preparation

32. Immediately after euthanasia, cell suspensions are obtained from one, or both, testes, exposed to hypotonic solution and fixed. The cells are then spread on slides and stained (7, 15). All slides should be blind coded so that their identity is not available to the scorer.

Analysis

33. At least 200 well spread metaphases should be scored for each animal (4). If the historical negative control frequency is < 1%, more than 200 cells/animal should be scored to increase the statistical power (4). Staining methods that permit the identification of the centromere should be used. Since fixation procedures often result in the breakage of a proportion of metaphase cells with loss of chromosomes, the cells scored should, therefore, contain a number of centromeres equal to the modal number $\pm$2.

34. Chromosome and chromatid-type aberrations should be recorded separately and classified by sub-types (breaks, exchanges). Gaps should be recorded, but not considered, when determining whether a compound induces significant increases in the incidence of cells with chromosomal aberrations. Procedures in use in the laboratory should ensure that analysis of chromosomal aberrations is performed by well-trained scorers and peer-reviewed as appropriate.

35. Although the purpose of the test is to detect structural chromosomal aberrations, it is important to record the frequencies of polyploid cells and cells with endoreduplicated chromosomes when these events are seen (see Paragraph 45).

DATA AND REPORTING

Treatment of results

36. Individual animal data should be presented in tabular form. For each animal the number of cells with structural chromosomal aberration(s) and the number of chromosome aberrations per cell should be evaluated. Chromatid- and chromosome-type aberrations classified by sub-types (breaks, exchanges) should be listed separately with their numbers and frequencies for experimental and control cultures. Gaps are recorded separately and reported but generally not included in the total aberration frequency. Percentage of polyploidy and cells with endoreduplicated chromosomes are reported when seen.

37. Data on toxicity and clinical signs as per Paragraph 11 should be reported.

Acceptability Criteria

38. 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 32).

b) Concurrent positive controls fulfil the positivity criteria (see Paragraphs 17, 18).

c) Adequate number of cells and doses have been analyzed (see Paragraphs 33 and 29).

d) The criteria for the selection of top dose are consistent with those described in Paragraphs 26, and 27.

39. If both mitosis and meiosis are observed, the ratio of spermatogonial mitoses to first and second meiotic metaphases should be determined as a measure of cytotoxicity for all treated and negative control animals in a total sample of 100 dividing cells per animal. If only mitosis is observed, the mitotic index should be determined in at least 1000 cells for each animal (see also Paragraph 6).

Evaluation and interpretation of results

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

41. 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. The test chemical is then considered able to induce chromosomal aberrations in spermatogonial cells of the test animals. Recommendations for the most appropriate statistical methods can also be found in the literature (4, 10). Statistical tests used should consider the animal as the experimental unit.

42. 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 chromosomal aberrations in the spermatogonial cells of the test animals. Recommendations for the most appropriate statistical methods can also be found in the literature (4, 12). A negative result does not exclude the possibility that the compound may induce chromosomal aberrations at later developmental phases not studied, or gene mutations. In the case of a negative result, the likelihood that the test chemical, or its metabolites, exposure the target tissue should be discussed.

43. 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 (9)

44. 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.

45. An increase in the number of polyploid cells may indicate that the test chemical has the potential to inhibit mitotic processes and to induce numerical chromosomal aberrations (17). An increase in the number of cells with endoreduplicated chromosomes may indicate that the test chemical has the potential to inhibit cell cycle progress (10, 11), which is a different mechanism of inducing numerical chromosome changes than inhibition of mitotic processes (see Paragraph 2). Therefore incidence of polyploid cells and cells with endoreduplicated chromosomes should be recorded separately.

Test report

The test report must include the following information:

Summary.

Test chemical:
- identification data and CAS no., 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;
- preparation of dietary, drinking water or inhalation formulations;
- analytical determinations on formulations (e.g., stability, homogeneity, nominal concentrations);
- solubility and stability of the test chemical in solvent/vehicle, if known.

Test animals:
- species/strain used;
- number and age 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 range finding study, if conducted;
- rationale for dose level selection;
- rationale for route of administration;
- details of test chemical preparation;
- details of the administration of the test chemical;
- rationale for sacrifice times;

- 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;
- detailed description of treatment and sampling schedules and justifications for the choices;
- method of euthanasia;
- procedures for isolating tissues;
- identity of metaphase arresting chemical, its concentration and duration of treatment;
- methods of slide preparation;
- criteria for scoring aberrations;
- number of cells analysed per animal;
- criteria for considering studies as positive, negative or equivocal.

Results:
- animal condition prior to and throughout the test period, including signs of toxicity;
- body and organ weights at sacrifice (if multiple treatments are employed, body weights taken during the treatment regimen);
- signs of toxicity;
- mitotic index;
- ratio of spermatogonial mitoses cells to first and second meiotic metaphases, or other evidence of exposure to the target tissue;
- type and number of aberrations, given separately for each animal;
- total number of aberrations per group;
- number of cells with aberrations per group;
- dose-response relationship, where possible;
- statistical analyses and methods applied;
- concurrent negative control data;
- historical negative control data with ranges, means, standard deviations, and 95% confidence interval (where available), or published historical negative control data used for acceptability of the test results;
- concurrent positive control data;
- changes in ploidy, if seen.

Discussion of the results.

Conclusion.

LITERATURE

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ANNEX

Definitions

Chromatid-type aberration: structural chromosome damage expressed as breakage of single chromatids or breakage and reunion between chromatids.

Chromosome-type aberration: structural chromosome damage expressed as breakage, or breakage and reunion, of both chromatids at an identical site.

Gap: an achromatic lesion smaller than the width of one chromatid, and with minimum misalignment of the chromatids.

Numerical abnormality: a change in the number of chromosomes from the normal number characteristic of the animals utilized.

Polyploidy: a multiple of the haploid chromosome number (*n*) other than the diploid number (i.e., 3*n*, 4*n* and so on).

Structural aberration: a change in chromosome structure detectable by microscopic examination of the metaphase stage of cell division, observed as deletions and fragments, exchanges.