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OECD GUIDELINE FOR THE TESTING OF CHEMICALS

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. This modified version of the Test Guideline reflects many years of experience with this assay and the potential for integrating or combining this test with other toxicity or genotoxicity studies. Combining toxicity studies has the potential to reduce the numbers of animals used in toxicity testing. This Test Guideline is part of a series of Test Guidelines on genetic toxicology. 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 *in vivo* mammalian spermatogonial chromosomal aberration test is to identify those chemicals that cause structural chromosomal aberrations in mammalian spermatogonial cells (2) (3) (4). In addition, this test is relevant to assessing genetoxicity because, although they may vary among species, factors of in vivo metabolism, pharmacokinetics and DNA-repair processes are active and contribute to the response. This guideline is not designed to measure numerical abnormalities; the assay is not routinely used for this purpose.

3. This test measures structural chromosomal aberrations (both chromosome- and chromatid-type) in dividing spermatogonial germ cells and is, therefore, expected to be predictive of induction of heritable mutations in these germ cells.

4. Definitions of key terms are set out in the Annex.

INITIAL CONSIDERATIONS

5. Rodents are routinely used in this test but other species may in some cases be appropriate if scientifically justified. Standard cytogenetic preparations of rodent testes generate mitotic (spermatogonia) and meiotic (spermatocyte) metaphases. Mitotic and meiotic metaphases are identified based on the morphology of the chromosomes (4). This *in vivo* cytogenetic test detects structural chromosomal 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 converted into chromosome-type-aberrations in subsequent cell divisions. Additional information from treated spermatocytes can be

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obtained by meiotic chromosome analysis for chromosomal structural aberrations at diakinesis-metaphase I and metaphase II.

7. A number of generations of spermatogonia are present in the testis (5), and these different germ cell types may have a spectrum of sensitivity to chemical treatment. Thus, the aberrations detected represent an aggregate response of treated spermatogonial cell populations. The majority of mitotic cells in testis preparations are B spermatogonia, which have a cell cycle of approximately 26 hr (3).

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, 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

10. Competency in this assay should be established by demonstrating the ability to reproduce expected results for structural chromosomal aberration frequencies in spermatogonia with positive control substances (including weak responses) such as those listed in Table 1 and obtaining negative control frequencies that are consistent with acceptable range of control data in the published literature (e.g. (2) (3) (6) (7) (8) (9) (10)) 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 young adult animals should be employed. Male mice are commonly used; however, males of other appropriate mammalian species may be used when scientifically justified and to allow this test to be run in conjunction with another Test Guideline. The scientific justification for using species other than rodents should be 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, 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) if no aggressive behaviour is expected, preferably in solid floor cages with appropriate environmental enrichment. Animals may be housed individually if scientifically justified.

Preparation of the animals

13. 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 using a humane, minimally invasive method (e.g. by ringing, tagging, micro-chipping or biometric identification, but not ear or toe 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 test chemical should be avoided. At the commencement of the study, the variation between individual animal weights should be minimal and not exceed ± 20%.

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 levels used, and should not be capable of chemical reaction with the test substances. 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. In the absence of historical or published control data showing that no structural chromosomal aberrations and other deleterious effects are induced by a chosen atypical solvent/vehicle, an initial study should be conducted in order to establish the acceptability of the solvent/vehicle control.

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). When a concurrent positive control group is not included, scoring controls (fixed and unstained slides) should be included in each experiment. These can be obtained by including within the scoring of the study appropriate reference samples that have been obtained and stored from a separate positive control experiment conducted periodically (e.g. every 6-18 months) in the laboratory where the test is performed; for example, during proficiency testing and on a regular basis thereafter, where necessary.

17. Positive control substances should reliably produce a detectable increase in the frequencies of cells with structural chromosomal aberrations over the spontaneous levels. Positive control doses should be chosen so that the effects are clear but do not immediately reveal the identity of the coded samples to the scorer. Examples of positive control substances are included in Table 1.

Table 1. Examples of positive control substances.

Substances [CAS No.] (reference no.)
Cyclophosphamide (monohydrate) [CAS no. 50-18-0 (CAS no. 6055-19-2)] (9)
Cyclohexylamine [CAS no. 108-91-8] (7)
Mitomycin C [CAS no. 50-07-7] (6)
Monomeric acrylamide [CAS 79-06-1] (10)
Triethylenemelamine [CAS 51-18-3] (8)

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. 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 control animals also should be included for every sampling time in order to establish acceptability of the vehicle control.

PROCEDURE

Number of animals

19. Group sizes at study initiation should be established with the aim of providing a minimum of 5 male animals per group. 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) (3) (11). As a guide to typical maximum animal requirements, a study at two sampling times with three dose groups and a concurrent negative control group, plus a positive control group (each composed of five animals per group), would require 45 animals.

Treatment schedule

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

21. In the highest dose group two sampling times after treatment are used. Since the time required for uptake and metabolism of the test substance(s), as well as its effect on cell cycle kinetics, can affect the optimum time for chromosomal aberration detection, 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 (less than or equal to the cell cycle time of B spermatogonia and thus optimizing the probability of scoring first post-treatment metaphases) after treatment should be taken, unless another sampling time is known to be more appropriate and justified.

22. Other sampling times may be used. For example in the case of chemicals that exert S-independent effects, earlier sampling times (i.e. less than 24 hr) may be appropriate.

23. 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. OECD 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.

24. 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 and rats, this interval is approximately 3 - 5 hours.

Dose levels

25. 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, and treatment regimen to be used in the main study, according to recommendations for conducting dose range-finding studies (12). This study should 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 euthanasia of the animals (13).

26. 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%.

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

28. In order to obtain dose response information, a complete study should include a negative control group (paragraph 18) and a minimum of three dose levels generally separated by a factor of 2, but by no 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 preferably cover a range from the maximum to a dose producing little or no toxicity. When target tissue (i.e. testis) toxicity is observed at all dose levels tested, further study at non-toxic doses is advisable. Studies intending to more fully characterize the quantitative dose-response information may require additional dose groups. For certain types of test substances (e.g. human pharmaceuticals) covered by specific requirements, these limits may vary. 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

29. The anticipated route of human exposure should be considered when designing an assay. Therefore, routes of exposure such as dietary, drinking water, topical subcutaneous, intravenous, oral (by gavage), inhalation, or implantation may be chosen as justified. In any case, the route should be chosen to ensure adequate exposure of the target tissue. Intraperitoneal injection is not normally recommended unless scientifically justified since it is not usually a physiologically relevant 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 to allow detection of the

effects (see paragraph 33). 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 body weight 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

30. 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, all animals should be observed for morbidity and mortality. All animals should be weighed at study initiation, at least once a week during repeated-dose studies, and at euthanasia. In studies of at least one-week duration, 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 (13).

Chromosome preparation

31. Immediately after euthanasia, germ cell suspensions are obtained from one, or both, testes, exposed to hypotonic solution and fixed following established protocols (e.g. (2) (14) (15). The cells are then spread on slides and stained (16) (17). All slides should be coded so that their identity is not available to the scorer.

Analysis

32. At least 200 well spread metaphases should be scored for each animal (3) (11). If the historical negative control frequency is < 1%, more than 200 cells/animal should be scored to increase the statistical power (3). Staining methods that permit the identification of the centromere should be used.

33. 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. Recognizing that slide preparation procedures often result in the breakage of a proportion of metaphases with a resulting loss of chromosomes, the cells scored should, therefore, contain a number of centromeres not less than $2n\pm2$, where n is the haploid number of chromosomes for that species.

34. 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 44).

DATA AND REPORTING

Treatment of results

35. 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 groups. Gaps

are recorded separately. The frequency of gaps is reported but generally not included in the analysis of the total structural chromosomal aberration frequency. Percentage of polyploidy and cells with endoreduplicated chromosomes are reported when seen.

36. Data on toxicity and clinical signs (as per Paragraph 30) 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, which are generally expected to be > 0% and ≤ 1.5% cells with chromosomal aberrations, 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 historical 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, 18).

c) Adequate numbers of cells and doses have been analyzed (see Paragraphs 28 and 32).

d) The criteria for the selection of top dose are consistent with those described in Paragraphs 25, and 26.

38. 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.

Evaluation and interpretation of results

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

40. 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 at least at one sampling time; and,

c. any of the results are outside 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 chromosomal aberrations in spermatogonial cells of the test animals. Recommendations for the most appropriate statistical methods can also be found in the literature (11) (18). Statistical tests used should consider the animal as the experimental unit.

41. 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 chromosomal aberrations in the spermatogonial cells of the test animals. Recommendations for the most appropriate statistical methods can also be found in the literature (11) (18). A negative result does not exclude the possibility that the compound may induce chromosomal aberrations at later developmental phases not studied, or gene mutations.

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

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 acceptable range of negative control data, or the laboratory's historical negative control data (19).

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 (20). An increase in the number of cells with endoreduplicated chromosomes may indicate that the test chemical has the potential to inhibit cell cycle progress (21) (22), 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

46. 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-constituents 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 solvent/vehicle.
- 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 use;
- number and age of animals;
- source, housing conditions, diet, etc.;
- method for uniquely identifying the animals
- for short-term studies: individual weight of the 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 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;
- method of analgesia (where used)
- 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 with means and standard deviations;
- number of cells with aberrations per group with means and standard deviations;
- 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, including frequencies of polyploidy and/or endoreduplicated cells.

Discussion of the results.

Conclusion.

LITERATURE

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ANNEX

Definitions

Aneuploidy**:** any deviation from the normal diploid (or haploid) number of chromosomes by a single chromosome or more than one, but not by entire set(s) of chromosomes (polyploidy).

Centromere: Region(s) of a chromosome with which spindle fibers are associated during cell division, allowing orderly movement of daughter chromosomes to the poles of the daughter cells.

Chromosome diversity: diversity of chromosome shapes (e.g. metacentrique, acrocentriques, etc….) and sizes.

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.

Clastogen**:** any substance which causes structural chromosomal aberrations in populations of cells or organisms.

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

Genotoxic: a general term encompassing all types of DNA or chromosome damage, including breaks, deletions, adducts, nucleotides modifications and linkages, rearrangements, mutations, chromosome aberrations, and aneuploidy. Not all types of genotoxic effects result in mutations or stable chromosome damage."

Mitotic index (MI): the ratio of cells in metaphase divided by the total number of cells observed in a population of cells; an indication of the degree of proliferation of that population.

Mitosis: division of the cell nucleus usually divided into prophase, prometaphase, metaphase, anaphase, and telophase.

Mutagenic: produces a heritable change of DNA base-pair sequence(s) in genes or of the structure of chromosomes (chromosome aberrations).

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.