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Recommendations for conducting the *in vivo* alkaline Comet assay

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The *in vivo* alkaline single cell gel electrophoresis assay, hereafter the Comet assay, can be used to investigate the genotoxicity of industrial chemicals, biocides, agrochemicals and pharmaceuticals. The major advantages of this assay include the relative ease of application to any tissue of interest, the detection of multiple classes of DNA damage and the generation of data at the level of the single cell. These features give the Comet assay potential advantages over other *in vivo* test methods, which are limited largely to proliferating cells and/or a single tissue. The Comet assay has demonstrated its reliability in many testing circumstances and is, in general, considered to be acceptable for regulatory purposes. However, despite the considerable data published on the *in vivo* Comet assay and the general agreement within the international scientific community over many protocol-related issues, it was felt that a document giving detailed practical guidance on the protocol required for regulatory acceptance of the assay was required. In a recent meeting held in conjunction with the 4th International Comet Assay Workshop (Ulm, Germany, 22–25 July 2001) an expert panel reviewed existing data and recent developments of the Comet assay with a view to developing such a document. This paper is intended to act as an update to the more general guidelines which were published as a result of the International Workshop on Genotoxicity Test Procedures. The recommendations are also seen as a major step towards gaining more formal regulatory acceptance of the Comet assay.

Introduction

The *in vivo* alkaline single cell gel electrophoresis assay, hereafter the Comet assay, can be used to investigate the genotoxicity of industrial chemicals, biocides, agrochemicals and pharmaceuticals. General review articles on the Comet assay include Tice (1995), Fairbairn *et al*. (1995), Anderson *et al*. (1998), Rojas *et al*. (1999), Speit and Hartmann (1999) and Singh (2000).

The basic principle of the Comet assay is the migration of DNA in an agarose matrix under electrophoretic conditions. When viewed under a microscope, a cell has the appearance of a comet, with a head (the nuclear region) and a tail containing DNA fragments or strands migrating in the direction of the anode. The detection of altered DNA migration is dependent on various parameters such as the concentration of agarose in the gel, the pH, temperature and duration of alkaline unwinding and the pH, temperature, voltage, amperage and duration of electrophoresis.

Among the various versions of the assay, the alkaline (pH of the unwinding and electrophoresis buffer > 13) method enables detection of the broadest spectrum of DNA damage and is, therefore, recommended (in the first instance) for regulatory purposes. It can detect double- and single-strand breaks, alkali-labile sites that are expressed as single-strand breaks and single-strand breaks associated with incomplete excision repair. Under certain conditions, the assay can also detect DNA–DNA and DNA–protein crosslinking, which (in the absence of other kinds of DNA lesions) appears as a relative decrease in DNA migration compared with concurrent controls.

The Comet assay can be applied to any tissue in the given *in vivo* model, provided that a single cell/nucleus suspension can be obtained. Therefore, the Comet assay has potential advantages over other *in vivo* genotoxicity test methods, which are reliably applicable to rapidly proliferating cells only (the cytogenetic evaluation of effects in bone marrow cells) and/or have been validated preferentially in a single tissue only (the liver unscheduled DNA synthesis assay). As such, its primary use is that of a supplemental assay for mechanistic investigations, similar to mutation analysis in transgenic systems. In particular, this seems important for investigation of suspected tissue-specific genotoxic activity, which includes 'site-of-contact' genotoxicity (cases of high local versus low systemic exposure).

Despite the plethora of data on the Comet assay and the overall agreement on a general protocol (see report of the IGWPT in Tice *et al.*, 2000), it was felt that a more formal document, giving recommendations on the conduct of the *in vivo* Comet assay for regulatory approval, was required. To this end an expert group met as part of the 4th International Comet Assay Workshop (Ulm, Germany, 22–25 July 2001) to review current protocols, identify areas which require more detailed guidance and produce a document similar to those prepared as part of the *UKEMS Guidelines* (Kirkland, 1990).

Materials and methods

Test animals

Rats or mice are preferred, although other mammalian species may be used when justified on the basis of existing data on structural analogues and/or from toxicokinetic, toxicodynamic studies. Laboratory strains commonly used in toxicity testing

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should be employed. To reduce inter-animal variability as much as possible, the weight variation of healthy, young adult animals at the commencement of treatment should not exceed ±20% of the mean weight for each sex.

The temperature in the experimental animal room should be 22°C (±3°C) and the relative humidity should be at least 30% and preferably not exceed 70%. Lighting should be artificial, with a 12 h light:12 h dark cycle. Water should be available *ad libitum* and the animals fed conventional laboratory diets. The animals should be acclimatized to the laboratory conditions for at least 5 days prior to treatment

Uniquely identified animals are randomly assigned to the control and treatment groups. Animals may be housed individually or be caged in small groups of the same sex, as appropriate, and the cages arranged in such a way that possible effects due to cage placement are minimized.

Test substance

Solid test substances should be dissolved or suspended in appropriate solvents or vehicles and diluted, if appropriate, prior to dosing of the animals. The solvent/vehicle should not produce toxic effects at the dose levels used and should not be suspected of chemical reactivity with the test substance. If other than well-known solvents/vehicles are used, their inclusion should be supported with reference data indicating their compatibility. Wherever possible, the use of an aqueous solvent/vehicle should be considered first.

Liquid test substances may be dosed directly or diluted prior to dosing. Fresh preparations of the test substance should be employed unless stability data demonstrate the acceptability of storage.

Controls

Concurrent positive and negative (solvent/vehicle) controls should be included for each sex in each independently performed part (e.g. sampling time) of the test. Except for treatment with the test substance, animals in the control groups should be handled in an identical manner to animals of the treatment groups.

The positive control should be an established genotoxic substance known to induce DNA damage detectable by the Comet assay. The positive control dose(s) should be chosen such that a positive response is obtained but does not immediately reveal the identity of the coded slides to the scorer. The positive response should not be associated with excessive target tissue cytotoxicity or with excessive animal toxicity. The positive control may be administered by a different route than the test substance, however, in the case of inhalation studies investigating effects on the respiratory tract, the positive control should be administered by the same route as the test substance. If technical competence in obtaining a positive response using positive controls in inhalation studies can be demonstrated, the positive control may be administered by a different route. The number of animals in the positive control group can be reduced when justified by the laboratory (e.g. by historical control data).

Examples of positive controls include: ethyl methanesulfonate (EMS) [CAS no. 62-50-0]; ethyl nitrosourea (ENU) [CAS no. 759-73-9]; methyl methanesulfonate (MMS) [CAS no. 66-27-3]; *N*-nitrosodimethylamine (N-DMA) [CAS no. 62-75-9]; 1-nitrosopiperidine [CAS no. 100-75-4].

Wherever possible, the use of a chemical class-related positive control substance should be considered.

Assay procedure

Study design

Using simulation studies, Wiklund and Agurell (2002) assessed the appropriateness of certain study design issues. It was demonstrated that groups of four animals provide comparable statistical power following analysis of 150 cells/animal (derived from three slides), compared with groups of five animals where analysis of 100 cells/animal (derived from two slides) is performed.

Animals

A minimum of four to five scorable animals per sex should be included in each dose group at each sample time. It is recommended that the total number of cells (and slides) scored per animal should be modified depending on the number of animals in each dose group. Fewer numbers of animals per sex and per dose group may be used with justification.

If, at the time of the investigation, there are data available from studies in the same species and using the same route of exposure that demonstrate that there are no substantial differences in toxicity between sexes, then testing in a single sex will be sufficient. Where human exposure to chemicals may be sex-specific, as for example with some pharmaceutical agents, the test should be performed with animals of the appropriate sex.

Treatment and sampling times

Either a single treatment or repeated treatments (generally at 24 h intervals) are equally acceptable. In both experimental designs, the study is acceptable as long as a positive effect has been demonstrated or, for a negative result, as long as an appropriate level of animal or tissue toxicity has been demonstrated or the limit dose with appropriate tissue exposure has been used. For repeated treatment schedules, dosing must be continued until the day of sampling. On a daily basis, test substances may be administered as a split dose (i.e. two treatments separated by no more than a few hours), to facilitate administering a large volume of material.

The test may be performed in two ways. (i) Animals are treated with the test substance once. In relation to the time of test substance administration, tissue/organ samples are obtained at 2–6 and 16–26 h after dosing. The shorter sampling time should be sufficient to detect rapidly absorbed as well as unstable or direct acting compounds; the late sampling time is to detect compounds which require time to be absorbed, distributed and metabolized. When a positive response is identified at one sampling time, data from the other sample time need not be collected. (ii) If multiple treatments at 24 h intervals are used, tissue/organ samples need be collected once only. The sampling time should be 2–6 h after the last administration of the test substance.

Alternative sampling times may be used when justified on the basis of toxicokinetic data.

Selection of dose levels

Generally, data from at least two dose levels are required. The highest dose level is defined as the dose producing signs of toxicity such that a higher dose level, based on the same dosing regimen, would be expected to produce mortality, an unacceptable level of animal toxicity or excessive cytotoxicity in the target tissue. In most studies, the low dose level should be 25–50% of the high dose. Substances with specific biological activities at low non-toxic doses (such as hormones and mitogens) may be exceptions to the dose setting criteria and

should be evaluated on a case-by-case basis. If a range-finding study is performed because no suitable toxicity data are available, it should be performed in the same laboratory, using the same species, strain, sex and treatment regimen to be used in the main study.

If a test at the limit dose produces no observable toxic effects and if genotoxicity would not be expected, based on data from structurally related substances, then a full study may not be necessary. The limit dose is 2000 mg/kg body wt/day for treatment up to 14 days and 1000 mg/kg body wt/day for treatment longer than 14 days. In this case, plasma exposure should be demonstrated. Expected human exposure levels may indicate the need for a higher dose level to be used in the limit test.

Administration of doses

The test substance is usually administered orally by gavage using a stomach tube or a suitable intubation cannula. The maximum volume of liquid that can be administered by gavage at one time should be based on the size of the test animal and should not exceed 20 ml/kg body wt. The use of higher volumes must be justified. Routes of exposure other than oral (e.g. dermal or inhalation) are acceptable, where justified. However, the i.p. route is not recommended when examining tissues such as the liver that could be exposed directly to the test substance rather than via the circulatory system. Inhalation exposures may be appropriate for testing gases, dusts, vapours or aerosols. Depending on the nature of the test substance and the tissue(s) to be sampled, either whole body or nose only exposure may be appropriate. With the exception of irritating or corrosive substances, which normally exacerbate effects at higher concentrations, variability in the test volume should be minimized by adjusting the concentration to ensure a constant volume at all dose levels.

Selection of the tissue(s)

The Comet assay can be applied to any tissue of the experimental animal, providing that a high quality single cell/nucleus suspension can be obtained. Selection of the tissue(s) to be evaluated should be based, wherever possible, on data from structural analogues, absorption, distribution, metabolism, excretion studies and/or other toxicological information. A tissue should not be evaluated unless there is evidence of, or support for, exposure of the tissue to the test substance and/or its metabolite(s). In the absence of such information and, unless scientifically justified, two tissues should be examined. Recommended tissues are liver, which is the major organ for the metabolism of absorbed compounds, and a site of first contact tissue, e.g. gastrointestinal for orally administered substances, respiratory tract for substances administered via inhalation or skin for dermally applied substances. Which tissue is evaluated first is at the discretion of the investigator and both tissues need not be evaluated if a positive response is obtained in the first tissue evaluated.

Preparation of single cells or cell nuclei

Single cell suspensions can be obtained from solid tissue by incubation with digestive enzymes such as collagenase or trypsin (for a review see Brendler-Schwaab *et al*., 1994), mincing briefly with a pair of fine scissors (Tice *et al*., 1991) or by pushing the tissue sample through a mesh membrane. Cell nuclei can be obtained by homogenization (Sasaki *et al*., 1997; Miyamae *et al*., 1998). During mincing or homogenization, EDTA can be added to the processing solution, to chelate calcium/magnesium and prevent endonuclease activation, and radical scavengers (e.g. DMSO) can be added, to prevent oxidant-induced DNA damage. Any cell dissociation method is acceptable as long as it can be demonstrated that the process is not associated with inappropriate background levels of DNA damage.

Cytotoxicity

Cell death is associated with increased levels of DNA strand breaks. In the Comet assay, the microscopical image resulting from necrotic or apoptotic cells are comets with small or non-existent head and large, diffuse tails (Olive *et al*., 1993; Fairbairn *et al*., 1996). These cells are commonly called 'hedgehogs', 'ghost cells', 'clouds' or 'non-detectable cell nuclei (NDCN)'. It was shown for *in vitro* tests that such cells can be present upon treatment with cytotoxic, non-genotoxic agents (Hartmann and Speit, 1997; Henderson *et al*., 1998). However, such microscopic images can also be seen after treatment with high doses of radiation or high concentrations of strong mutagens, indicating that such comets are not uniquely diagnostic for apoptosis/necrosis. It is yet not clear whether increased DNA fragmentation due to cell death can result in the generation of false positive results in the Comet assay. *In vitro* data suggest that false positive results due to cytotoxicity may be cell type-specific. Excessive cytotoxicity in V79 Chinese hamster cells (Hartmann *et al*., 2001), L5178Y mouse lymphoma cells (Kiskinis *et al*., 2002) and colon cells (Roser *et al*., 2001) did not lead to positive results in the Comet assay. Furthermore, in primary rat hepatocytes used for *in vitro* treatment, no differences in tail length were seen between cultures in which viability (as measured by Trypan blue exclusion) ranged from 86 to 58% (Frei *et al*., 2001). In contrast, cytotoxicity was reported as a possible confounding effect in TK-6 cells (Henderson *et al*., 1998), rat lymphocytes (Quintana *et al*., 2000) and Jurkat cells (Choucroun *et al*., 2001).

No data are yet available on whether cell death in tissues of experimental animals may also be associated with increased DNA migration patterns in the Comet assay *in vivo*. Thus, the expert group recommends a concurrent assessment of cytotoxicity for the correct interpretation of Comet assay data. The extent of necrosis or apoptosis in tissues could be determined using histopathology. It is suggested that sections of the target tissue are made and, in the light of a positive Comet response, the tissue is investigated for evidence of necrosis or apoptosis. Simple dye exclusion methods for assessing cytotoxicity (e.g. Trypan blue exclusion) are only adequate for methods of cell isolation where membrane integrity is not affected (e.g. for hepatocytes derived from liver perfusion; Frei *et al*., 2001). A dual dye viability assay based on a combination of 5,6-carboxyfluorescein diacetate and ethidium bromide (Strauss, 1991) may have the advantage of providing some information on the metabolic activity of the cell being still present. A further possibility for assessing the occurrence of apoptotic/necrotic cells is to process additional Comet assay slides under modified conditions. Due to the very low molecular weight of the DNA in apoptotic/necrotic cells, these cells may be lost under electrophoretic conditions and lead to an underestimate of the proportion of apoptotic/necrotic cells. By analysis of slides after lysis without further alkaline unwinding or electrophoresis (Vasquez and Tice, 1997) or by omitting the electrophoresis step after alkaline unwinding (Godard *et al*., 1999), cells with low molecular weight DNA

resulting from degradation due to double-strand breaks can be detected. This method might, therefore, provide valuable information for the interpretation of a positive response.

Given the uncertainties described, the expert group felt that certain cytotoxicity threshold values cannot be recommended because of lack of *in vivo* data and the inconsistency of *in vitro* data. However, including apoptotic/necrotic cells in the analysis could result in false positives. It is, therefore, important to identify apoptotic/necrotic cells by means of their images as described above and exclude them from the analysis. In addition, the intercellular distribution of comet response should be determined, which can provide important information: a bimodal distribution of cells with control level DNA migration and high levels of DNA migration only may suggest a cytotoxic rather than a genotoxic response, whereas in the latter case one would expect a dose-dependent increase in DNA migration in most, if not all, of the cells.

Slide preparation and processing

General

The basic steps of the assay include preparation of microscopic slides layered with cells embedded in an agarose gel, lysis of cells to liberate the DNA, DNA unwinding, electrophoresis, neutralization of the alkali, an optional alcohol treatment and drying step, DNA staining and scoring. These steps should be performed in a consistent manner that allows for the reliable detection of genotoxic agents. As a general rule, all buffers and reagents used in the assay should be given shelf-lives of no more than 1 month. The assay should be performed such that cells from control animals exhibit some level of DNA migration to enable the detection of crosslinks.

Slide preparation

Gel retention on slides is critical and although fully frosted slides exhibit increased gel bonding and thus gel stability, conventional microscope slides are also commonly used. These have the major advantage that the agarose gels can be dried and stored until scored and then subsequently archived (see Klaude *et al*., 1996). The number of agarose layers used per gel ranges from one to three. When multiple layers are used, the bottom layer generally consists of 1.0–1.5% agarose and may be dried prior to the addition of the cell-containing layer. The second or cell-containing layer is generally prepared from low melting point (LMP) agarose at 0.5–1.0%. There is an optional additional layer of LMP agarose at the same concentration as the cell-containing layer, although it is generally felt that this third layer is redundant. The concentration of cells in agarose, as well as the concentration of agarose, are important parameters for ensuring a successful analysis. High cell densities can result in a significant proportion of overlapping comets, especially at high levels of DNA migration. Higher agarose concentrations can affect the extent of DNA migration, i.e. could lead to limited DNA migration.

Lysis

After the agarose gel has solidified, the slides are placed, generally for at minimum of 1 h, in a lysis solution consisting of high salts and detergents. The recommended lysing solution consists of 100 mM EDTA, 2.5 M sodium chloride and 10 mM Trizma base, adjusted to pH 10.0, with 1% Triton X-100 added just prior to use. The addition of 1% *N*-lauroylsarcosine is now considered redundant. Addition of 10% DMSO to the lysing solution may be useful to prevent potential radical-induced DNA damage associated with the iron released during lysis from erythrocytes present in blood and tissue samples. The lysing solution is chilled prior to use, primarily to maintain the stability of the agarose gel. In order to standardize the content of the alkaline buffer and electrophoresis buffer after lysis, it is recommended that the gels be rinsed in water to remove residual detergents and salts prior to the alkali unwinding step, since the concentration of sodium chloride in the electrophoresis buffer may have an influence on electrophoresis conditions (Klaude *et al*., 1996).

Unwinding

Prior to electrophoresis, the slides are incubated in alkaline electrophoresis buffer to produce single-stranded DNA and to express alkali-labile sites as single-strand breaks. The recommended alkaline solution consists of 1 mM EDTA and 300 mM sodium hydroxide, pH $>$ 13. This solution maximizes the expression of alkali-labile sites as single-strand breaks. The length of time used for unwinding varies, mainly depending on the cell type used. An unwinding time of 20 min is sufficient to detect the presence of alkali-labile sites; other times can be used with justification. The temperature of the unwinding solution should be kept constant to minimize assay variability.

Electrophoresis conditions

Following alkali unwinding and expression of alkali-labile sites, the single-stranded DNA in the gels is electrophoresed under alkaline (pH $>$ 13) conditions to produce comets. Due to the large variability in the size of commercially available electrophoresis units, the voltage should be given as V/cm, ranging from 0.7 to 1.0 V/cm, with an accompanying amperage of ~300 mA, and the same electrophoresis unit and power supply should be used throughout a study. Slide-to-slide variation can be further minimized by maintaining a constant temperature during electrophoresis (generally between 2 and 20°C, although ~5°C is recommended) and by using a recirculating electrophoresis unit. Once the electrophoretic conditions have been established, the optimal electrophoresis duration depends on the extent of DNA migration desired in control cells, the range of responses being evaluated in cells from treated animals, the type of cell being investigated and the electrophoretic equipment used. In all cases, the conditions must be such that the DNA from the control cells exhibits some migration. The extent of migration among control cells can also be a critical issue in situations where DNA–DNA or DNA–protein crosslinking is induced. DNA crosslinking can be detected by the Comet assay, as demonstrated by retardation in the degree of DNA migration. Assay variability may be reduced by ensuring that replicate slides are scored for each tissue sample and that slides are randomly distributed in a gel box or among multiple electrophoretic runs in a fully balanced design.

Neutralization

After electrophoresis, the alkali in the gels is neutralized by rinsing the slides with a suitable buffer (e.g. Trizma base at pH 7.5). After neutralization, slides can be stained and comets scored or the gel can be dried, the slides stored and the comets scored when convenient. In the latter case, the agarose gels can be dehydrated by immersing the slides in absolute ethanol or methanol for a brief time (e.g. 2 min) or by letting the slides dry at room temperature (Klaude *et al*., 1996).

DNA staining and comet visualization

The DNA-specific dye and the magnification used for comet visualization depend largely on investigator-specific needs and the method of scoring. The fluorescent dyes used most frequently are ethidium bromide, propidium iodide, 4,6-diamidino-2-phenylindole (DAPI), SYBR Green I and YOYO-1 (benzoxazolium-4-quinolinum oxazole yellow homodimer). Antifade can be used for some fluorescent dyes to minimize the rate of signal quenching (Hartmann *et al.*, 2001). Non-fluorescent techniques for visualizing comets based on staining with silver nitrate have also been used. Which magnification is most appropriate depends on the type of cell being evaluated, the range of migration responses to be measured and the constraints of the microscope and/or imaging system. The most common magnifications used have been between 200× and 400×.

Analysis

The slide preparations should contain sufficient cells and be of adequate quality to permit a meaningful assessment of DNA damage.

Slides should be coded before analysis unless fully automated analysis is used. As a minimum, 100 comets should be scored per tissue per animal, with 50 comets scored per replicate slide when five animals are used per group. When using four animals per group, three slides per tissue should be scored with 50 cells per slide (Wiklund and Agurell, 2002).

DNA migration can be determined visually by the categorization of comets into different 'classes' of migration (Collins *et al*., 1993) or by using an eyepiece micrometer to estimate image or tail length. However, image analysis is recommended, with the measurement of parameters such as the percentage of DNA in the tail (per cent migrated DNA), tail length and tail moment (fraction of migrated DNA multiplied by some measure of tail length). Of these, tail moment and/or tail length measurements are the most commonly reported, but there is much to recommend the use of per cent DNA in tail, as this gives a clear indication of the appearance of the comets and, in addition, is linearly related to the DNA break frequency over a wide range of levels of damage. The approach or parameter used must be clearly defined and, if not typical, be justified.

Data and reporting

Treatment of results

Individual animal data and group summaries should be provided in tabular form. The intercellular distribution of comet responses within each animal is helpful for data interpretation, since this allows for the detection of sub-populations within the preparation. Also, a selected group of representative images may be provided to support the conclusions made. The mean extent of DNA migration and an associated error term should be calculated for each animal and for each dose group. Concurrent measures of cytotoxicity for each animal in all dose groups, including the solvent control animals, should be presented when a positive result has been reported for the test compound. Negative findings do not require reporting of cytotoxicity assessment as long as the limit dose was used or the highest dose tested was based on animal toxicity.

Definition of a positive response

A positive response is defined as one in which there is:

(i) a dose-related change in the defined measurement, e.g. tail length, moment, etc., between the control and test groups at least at a single sampling time; or

(ii) a change in the defined measurement, e.g. tail length, moment, etc., in a single dose group at least at a single sampling time.

A change in the defined measurement could be determined either by a comparison with a threshold value or by using statistical methods. The unit to be used for analysis of data is the animal. The threshold of a positive response could be defined as a simple multiple of the control measurement and this must be clearly stated and justified with reference to historical control data. Statistical methods may also be used as an aid in evaluating the test results (Lovell *et al.*, 1999; Wiklund and Agurell, 2002) and these must take account of the hierarchical nature of the data, i.e. slide/animal/group. Data presented to the expert group showed that more statistical power was achieved if the tail moment and tail length measures were log transformed (Wiklund and Agurell, 2002).

Biological relevance of data should be considered, i.e. parameters such as magnitude of response, inter-animal variation, dose–response relationship and cytotoxicity should be taken into account. Where a positive response is obtained it is critical that the investigator(s) assess the possibility that a cytotoxic rather than a genotoxic effect was responsible. Information on the extent of cytotoxicity associated with each positive dose group, the nature of the dose–response curve and the presence or absence of DNA double-strand breaks in the treated cell population may be useful in this regard. The intercellular distribution of comet response at each dose may provide important information: a bimodal distribution of cells with low and high levels of DNA migration only may suggest a cytotoxic response.

Although most experiments will give clearly positive or negative results, in rare cases the data set will preclude making a definite judgement about the activity of the test substance. Therefore, results may remain equivocal or questionable regardless of the number of times the experiment is performed. In such cases the suitability of the Comet assay or the species/target organ(s) evaluated must be questioned and other strategies investigated.

Positive results indicate that the test substance induces DNA damage *in vivo* in the target tissue(s) investigated. Negative results indicate that, under the test conditions used, the test substance does not induce DNA damage *in vivo* in the tissue(s) evaluated.

Information to be reported in the test report

The test report must include the following information.

Introductory information: the rationale for using the Comet assay and the experience of the laboratory with the methodology. This must include a definition of the parameters to be measured, e.g. tail length moment, etc., and what would constitute a positive result. The definition must clearly define the threshold for a positive and why it was chosen or the statistical method(s) used to evaluate the data.

Test substance: identification and CAS number, if known; physical nature and purity; physicochemical properties relevant to the conduct of the study; stability of the test substance, if known.

Solvent/vehicle: justification of the choice of solvent/vehicle;

solubility and stability of the test substance in the solvent/vehicle, if known.

Test animals: species/strain used; 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; choice of tissue(s) and justification.

Test conditions: positive and negative vehicle/solvent controls; data from a range-finding study, if conducted; rationale for dose level selection; likelihood that the test substance or its metabolites reached the general circulation or specifically the target tissue(s); details of test substance preparations; details of the administration of the test substance; rationale for route of administration; methods for verifying that the test agent reached the general circulation or target tissue, if applicable; conversion from diet/drinking water test substance concentration (p.p.m.) to the actual dose (mg/kg body wt/day), if applicable; details of food and water quality; a detailed description of the treatment and sampling schedules; methods for measurement of toxicity; detailed methods of single cell/nucleus preparation; methods of slide preparation, including agarose concentration, lysis conditions, alkali conditions and pH, alkali unwinding time and temperature, electrophoresis conditions (pH, V/cm, mA and temperature) and staining procedure; criteria for scoring comets and number of comets analysed per slide, per tissue and per animal; evaluation criteria; criteria for considering studies as positive, negative or equivocal.

Results: individual tissue, animal and group mean values for DNA migration; dose–response relationship, if available; statistical evaluation; signs of toxicity or cytotoxicity in the appropriate tissue(s); concurrent negative (solvent/vehicle) and positive control data; historical negative (solvent/vehicle) and positive control data with range, means and standard deviations; viability of the cells, if applicable.

Discussion of the Results, including reference to historical control data.

Conclusion: negative, positive or equivocal.

Concluding remarks

It is the nature of the Comet assay that several sources of variability exist (e.g. animal-to-animal, slide-to-slide, cell-to-cell and position of the slide in the electrophoresis box) which can have an impact on the result and which have to be accounted for in the design of a study. In the present recommendations, minimal standards for obtaining reproducible and reliable Comet assay data were identified which slightly deviate from those concluded following the IWGTP meeting in Washington in 1999 (Tice *et al*., 2000).

Experimental variability is an important issue as identified by the expert panel. This guidance document focuses in part on this aspect and recommendations are made to reduce experimental variability to ensure more reliable interpretation and better comparability of the data obtained with *in vivo* Comet experiments. The expert panel concluded that some publications would not fully meet what was agreed upon as requirements for an acceptable test. Some of this criticism applies, for example, to the most comprehensive overview on *in vivo* Comet assay test results published so far, by Sasaki *et al*. (2000). This paper provides data on more than 200 compounds tested in rodents, all of which were produced before the recommendations by Tice *et al.* (2000) were available. Therefore, although a valuable resource with very relevant data, the paper by Sasaki *et al.* (2000) has areas where technical aspects of the assay differ from the minimal requirements as identified in the present recommendations, as well as from the recommendations given by Tice *et al.* (2000). These aspects include: number of doses tested, number of animals per dose, slides per animal, number of cells, sufficient DNA migration in cells of concurrent controls and minimum time for treatment of slides with alkaline buffer. Considering these discrepancies, the data published by Sasaki *et al*. (2000), as well as papers not in agreement with the present recommendations, should be interpreted with caution.

The present recommendations represent a work in progress. Still, appropriately designed multi-laboratory international validation studies are necessary to gather information on additional potential sources of variability and on the intra- and inter-laboratory reproducibility of Comet assay results. It is hoped that the present guidance is a major step towards gaining more formal regulatory acceptance of the Comet assay and will also serve as the basis for further developments of this assay.

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