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The In Vitro Alkaline Comet Assay in Genetic Toxicology

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Abstract

The single cell gel (SCG) electrophoresis or comet assay is a technique detecting of multiple classes of DNA damage such as single and double strand breaks and oxidative base damage and is used for a wide range of applications. The comet assay is a relatively sensitive test which enables to measuring DNA damage spontaneously and caused by various agents. The principle advantages of the technique are apply to any cell type from prokaryotic and eukaryotic organisms, cultured cells or cells isolated from the organisms and it. The other advantages are its reliability, low costs, ease of application, and the short time needed to complete a study. Therefore, it has gained in popularity as a standard technique for evaluation of DNA damage/repair, biomonitoring and genotoxicity studies. The basic steps of the comet assay are preparation of microscope slides layered with cells in agarose, lysis, alkali treatment, electrophoresis under alkaline conditions, neutralization, DNA staining, comet visualization and comet scoring. Image analysis tools are available to analyze various parameters of the comets. The aim of this review is to give information about the principles and applications of the comet assay, a sensitive and rapid technique for the measurement of DNA damage.

Key words: Single cell gel electrophoresis, comet assay, DNA damage, genotoxicity.

INTRODUCTION

Genetic toxicology studies are conducted early in the safety testing program of many hazardous agents. Thus, it plays a dual role safety evaluation programs and with each passing day, it is gain ground. The comet assay, also called single cell gel electrophoresis (SCGE) is one of the genotoxicty tests and is a method detecting DNA fragmentation can be caused by various chemical or physical agents. This method can be used to investigate the genotoxicity of industrial chemicals, biocides, agrochemicals and pharmaceuticals. As a test for genotoxicity, the comet assay can be also used to identify possible human mutagens and carcinogens and DNA repair in cancer patient [1-13].

DNA damage and its repair in single-cell suspensions prepared from yeast, protozoa, plants, invertebrates and mammals can also be studied using the comet assay. Originally developed to measure variation in DNA damage and repair capacity within a population of mammalian cells, applications of the comet assay now range from human and sentinel animal biomonitoring to measurement of DNA damage in specific genomic sequences [14]. Much of the interest in this method comes from its potential applications in human biomonitoring and in ecological assessment of sentinel organisms exposed to environmental contaminants. Recently it is placed also in research of aging, molecular epidemiology, apoptosis, nutritional DNA damage, oxidative stress-antioxidants, cancer, chemotherapy and radiotherapy. Therefore, the technique that enables the sensitive measurement of DNA damage has become important recently [8, 10, 12, 13, 15-26]. Valverde and Rojas [27] reported 122 studies were available from the National Center for Biotechnology Information (NCBI) database that analyzed occupational and environmental exposure of human populations using the alkaline comet assay.

The concept of microgel electrophoresis was first introduced by Ostling and Johanson [28] as a method to measure DNA single-strand breaks. A modified version was developed by Singh et al. [29], which used alkaline electrophoresis (pH >13) to detect DNA damage induced by physical agents and chemicals [14]. This version, which is capable of detecting single-strand DNA breaks, alkali labile sites, crosslinks, and incomplete DNA repair sites in individual cells, is known as the ‘‘single cell gel’’ (SCG) electrophoresis technique. Subsequently, Olive et al. [30, 31] adapted the neutral technique of Ostling and Johanson [28] so that the comet assay could include lysis of cells by alkali treatment followed by electrophoresis at either neutral or mild alkaline (pH 12.3) conditions to detect single-strand DNA breaks. The Singh et al. [29] and Olive and Banath [14] methods are identical in principle and similar in practice, but the Singh method appears to be at least one or two orders of magnitude more sensitive in detecting DNA damage depending on the agent [27].

The alkaline comet assay is known as a rapid, simple and sensitive method for measuring and analyzing DNA single strand breaks and alkali labile sites [1, 6, 19, 29, 32, 33]. Compared with other genotoxicity assays, the advantages of the technique include: (a) its demonstrated sensitivity for detecting low levels of DNA damage; (b) its applicability to various tissues and/or special cell types (c) the requirement for small numbers of cells per sample; (d) sensitivity; (e) low costs; (f) ease of application and test performance; (g) the ability to conduct studies using relatively small amounts of a test substance; and (h) the relatively short time period (a few days) needed to complete an experiment [8, 18, 22, 24, 33-35].

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 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 appears as a relative decrease in DNA migration compared with concurrent controls [10].

METHODOLOGY

All methodological steps associated with the alkaline (pH>13) comet assay are equally important for obtaining reproducible and reliable results. In general, best results are obtained if sample processing, solution preparation and usage, and equipment utilization and maintenance are conducted using the strict quality control criteria considered appropriate for techniques in molecular biology [8].

The basic principle of the comet assay is the migration of DNA in an agarose matrix under electrophoretic conditions. Once a suspension of cells is obtained, the basic steps of the assay include preparation of microscope slides layered with cells in agarose; lysis of cells to liberate DNA; exposure to alkali (pH>13) to obtain single-stranded DNA and to express ALS as SSB; electrophoresis under alkaline (pH>13) conditions; neutralization of alkali; DNA staining and comet visualization and comet scoring (Figure 1) [8, 10].

Cell Types

Any eukaryote cell can theoretically be used for genotoxicity testing in the comet assay. However, generally, well-characterized cell lines or primary cells used generally in genetic toxicology testing for assessing other types of genetic damage (e.g., chromosomal aberrations, micronuclei, and mutations) are preferred. Standard cell lines include mouse lymphoma L5178Y, Chinese hamster ovary and Chinese hamster lung; standard primary cells include human lymphocytes and rodent hepatocytes [8, 24, 36, 37].

Media Conditions

Appropriate culture media and incubation conditions (culture vessels, CO2 concentration, temperature, and humidity) should be used in maintaining cultures. Although there are no data to support this requirement, established cell lines should be checked routinely for mycoplasma contamination and should not be used if contaminated [8].

Culture Preparation

For established cell lines, cells are propagated from stock cultures, and incubated in appropriate culture medium at 37°C. Lymphocytes isolated from the whole blood of healthy subjects are incubated in appropriate culture medium at 37°C. Rodent hepatocytes are isolated using routine procedures and incubated in appropriate culture medium at 37°C [8].

Treatment with Test Substance and Groups

The test substances should be dissolved or suspended in appropriate solvents or vehicles and diluted. In addition to the test substance group, positive and negative (solvent and/or vehicle) controls must be included in each experiment [8].

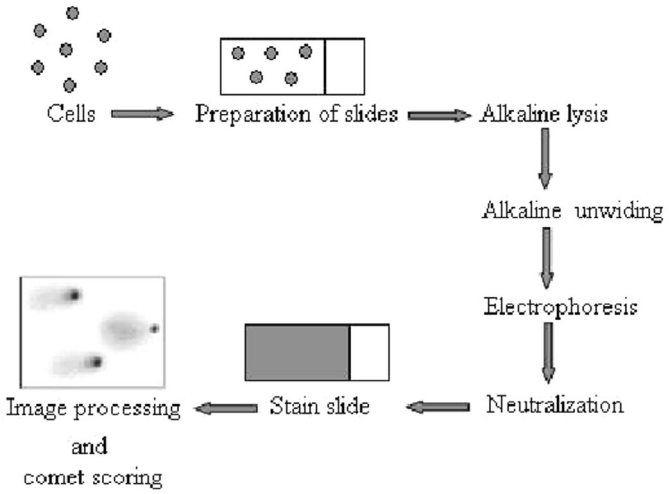

Figure 1. Schematic representation of critical steps in the alkaline comet assay.

PROCEDURE

Preparation of Slides

A number of different techniques have been used to prepare comet slides. Generally, but not exclusively, microscope slides are used, with each slide containing one or two independent gels. Initially, fully frosted slides were used most commonly because they offered increased gel bonding and thus stability. However, within the last few years, either conventional microscope slides or slides specifically modified to increase gel stability have been used increasingly. These have the major advantage that the agarose gels can be dried and stored until scored and then subsequently archived [8, 10, 38]. The number of agarose layers used per gel ranges from one to three. Cells are suspended in low melting point (LMP) agarose (generally at 37°C) and placed directly on a slide. Slides can be prepared ahead of time and stored with desiccant [8, 10].

Lysis

After the agarose gel has solidified, the slides are placed, generally for at minimum of 1 hour, in a lysis solution consisting of high salts and detergents. The original lysing solution developed by Singh et al. [29] consisted of 100 mM ethylene diamine tetra acetic acid (EDTA), 2.5 M sodium chloride, 1% N-lauroylsarcosine, 10 mM Trizma base, adjusted to pH 10.0, with 1% Triton X-100 added just prior to use. Tice et al. [39] added 10% dimethyl sulfoxide (DMSO) to the lysing solution to prevent radical-induced DNA damage associated with the iron released during lysis from erythrocytes present in blood and tissue samples. More recently, McKelvey-Martin et al. [32] have reported that N-laurylsarcosine is not needed in the lysing solution (i.e., that the inclusion of Triton X-100 is sufficient for cell lysis). The lysing solution is chilled prior to use, primarily to maintain the stability of the agarose gel. There is a minimal time needed to appropriately liberate the DNA and this time might vary depending on the cell type. The lysis duration used by different investigators varies considerable, from less than 1 hour to weeks if not months. 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. Besides between lysis and alkali unwinding, the liberated DNA can be incubated with proteinase K (PK) to remove residual protein or probed with DNA repair enzymes/antibodies to identify specific classes of DNA damage [8, 10, 14, 38].

Alkali (pH > 13) 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 minute 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 [8, 10, 12, 29].

Electrophoresis

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. The electrophoretic conditions developed by Singh et al. [29] were 25 V and 300 mA, with the DNA being electrophoresed for 20 minute. 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 accompanying amperage of 300 mA, and the same electrophoresis unit and power supply should be used throughout a study. Electrophoresis has been conducted at temperatures ranging from 5°C to room temperature. The reported electrophoresis duration has generally ranged from 5 to 40 minute, depending on the type of cell being used and the purpose of the experiment [8, 10, 29, 32].

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) at least 5 minutes. 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 cold absolute ethanol or methanol for a brief time (e.g. 2 min) or by letting the slides dry at room temperature [8, 10, 29, 38].

DNA Staining

The DNA-specific dye and the magnification used for comet visualization depend largely on investigator-specific needs and presumably have little effect on assay sensitivity or reliability [8, 10]. 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) [28, 29, 40-43].

Comet Visualization, Scoring and Analysis

All slides, including those of the positive and negative controls, should be independently coded before microscopic analysis and scored without knowledge of the code. Comet image magnification has generally varied from 160X to 600X, with 200X to 400X being used most commonly. Selection as to which magnification is most appropriate depends on the type of cell being evaluated, the range of migration responses to measure, and the constraints of the microscope and/or imaging system. However, the most common magnifications used have been between 200X and 400X. Generally, 50 to 100 randomly selected cells are analyzed per sample. 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 (Figure 2). Cells with an increased frequency of DNA strand breaks displayed increasing the amount of DNA in the comet tail (Figure 3). 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 [8, 10, 12, 24, 44].

The most flexible approach for collecting comet data involves the application of image analysis techniques to individual cells, and several dedicated software programs are commercially available. Moreover, a fully automated comet analysis imaging system has been developed [8, 45, 46]. As the use of computerized image analysis systems to collect comet data has increased, a metric based on the percentage of migrated DNA has become used more frequently [8, 31]. Using image analysis software, individual comet images were analyzed for several measurements including total intensity (DNA content), head and tail length, percent DNA in tail and tail moment which is fraction of migrated DNA multiplied by some measure of tail length) (Figure 4) [8, 14, 42, 47, 48]. 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 [10].

The experimental unit of exposure for in vitro studies is the culture, and all statistical analyses should be based on the individual culture response. The mean extent of DNA migration and an associated error term should be calculated for each dose group, as well as for each culture within a dose group. Concurrent measures of cytotoxicity for all cultures and dose groups, including the negative and positive controls, should be included [8, 24, 49, 50].

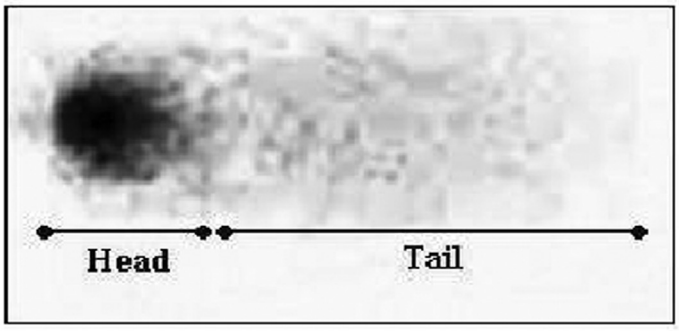

Figure 2. An illustration of the cell DNA migration with the comet assay.

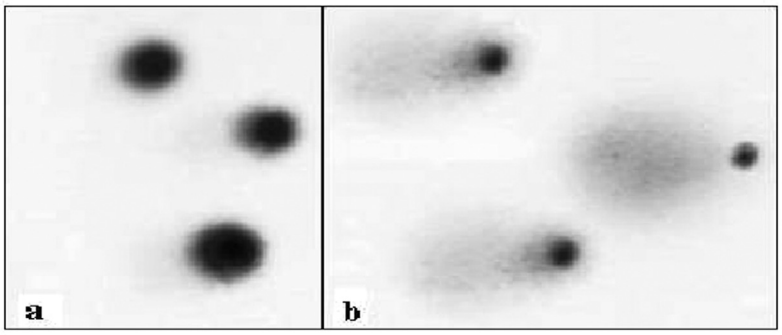

Figure 3. An illustration of nucleus with undamaged DNA (a) and nucleus with undamaged DNA (b) with the comet assay.

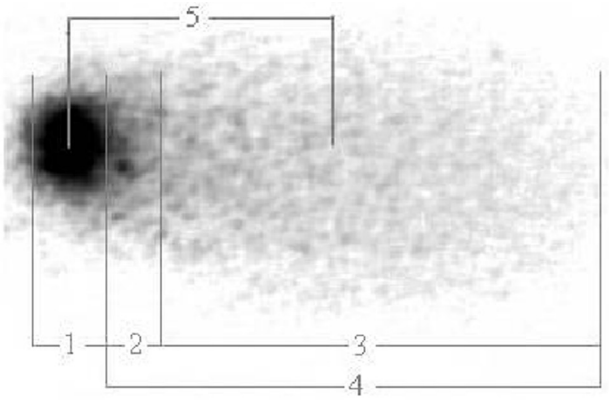

Figure 4. The measurements used for comet images.
1: Head length, 2: Body length, 3: Tail length, 4: Tail length without body, 5: Tail moment length

CONCLUSION

DNA damage may be resulted either from spontaneously or effects of various environmental factors and caused to serious results. Techniques of the measurement of DNA damage have become very important recently because of the increases of genotoxic, mutagenic and carcinogenic agents. The comet assay offers a useful screening test for possible cytotoxic and genotoxic effects. The comet assay has many advantages and it is early detect DNA damage and the amount of damage on single cell level. Therefore, the advantages of the comet assay provide the researcher with a versatile and powerful tool for genetic toxicology studies. It is hoped that the comet assay will gain more formal regulatory acceptance and will also serve as the basis for further developments of this assay.

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