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Review

Measuring oxidative damage to DNA and its repair with the comet assay ☆

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ABSTRACT

Background: Single cell gel electrophoresis, or the comet assay, was devised as a sensitive method for detecting DNA strand breaks, at the level of individual cells. A simple modification, incorporating a digestion of DNA with a lesion-specific endonuclease, makes it possible to measure oxidised bases.
Scope of review: With the inclusion of formamidopyrimidine DNA glycosylase to recognise oxidised purines, or Nth (endonuclease III) to detect oxidised pyrimidines, the comet assay has been used extensively in human biomonitoring to monitor oxidative stress, usually in peripheral blood mononuclear cells.
Major conclusions: There is evidence to suggest that the enzymic approach is more accurate than chromatographic methods, when applied to low background levels of base oxidation. However, there are potential problems of over-estimation (because the enzymes are not completely specific) or under-estimation (failure to detect lesions that are close together). Attempts have been made to improve the inter-laboratory reproducibility of the comet assay.
General significance: In addition to measuring DNA damage, the assay can be used to monitor the cellular or in vitro repair of strand breaks or oxidised bases. It also has applications in assessing the antioxidant status of cells. In its various forms, the comet assay is now an invaluable tool in human biomonitoring and genotoxicity testing. This article is part of a special issue entitled current methods to study reactive oxygen species — pros and cons.

1. Introduction: the comet assay

The comet assay was introduced almost 30 years ago [1,2] as a simple way of detecting DNA breaks. Cells are embedded in agarose, lysed, and electrophoresed at high pH; DNA containing breaks is drawn towards the anode, forming a comet like image when viewed by fluorescence microscopy (Fig. 1). With modifications, the comet assay has become the most popular method for measuring DNA damage of various sorts, including oxidative damage inflicted by reactive oxygen species (ROS). Applications include genotoxicity testing, human biomonitoring, ecogenotoxicology as well as basic research on DNA damage and repair.

Breaks are detected at the level of individual cells, and so the prime requirement is for a suspension of single cells, in as near a pristine state as possible. The assay can be applied to cultured mammalian cells, peripheral blood mononuclear (PBMN) cells, disaggregated tissues, haemolymph from molluscs, yeast, and nuclei isolated from plant tissue by chopping with a sharp blade. It is common to cryopreserve cells — particularly PBMN cells from biomonitoring studies, so that samples can be analysed in batches at a later date. Cryopreservation is done by controlled slow freezing of cells to −80 °C in medium containing dimethylsulphoxide, which prevents shearing of DNA by ice crystal formation. Too rapid centrifugation can also cause DNA breaks, as can over-trypsinisation of cells in monolayer culture.

The cell suspension is mixed with low melting point agarose at 37 °C, quickly spread on a microscope slide, covered with a cover slip, and chilled on ice to form a thin gel. Alternatively, in recently described methods designed for higher throughput [3,4], drops of a few μl of the agarose–cell mixture are placed on the chilled slide, or GelBond film, where the mini-gels set instantly (without cover slips).

The slides (minus cover slips) are placed in a lysis solution containing high salt and a detergent. Together, these remove membranes, allowing soluble cell and nuclear components to diffuse away, and strip histones from the DNA. The residual structures, containing highly condensed DNA, still resemble nuclei but are now known as nucleoids. The slides are placed in a solution of 0.3 M NaOH with EDTA, pH > 13, for a period of around 20–40 min, and then electrophoresed, typically for 20–30 min at a voltage gradient around 1 V per cm over the platform holding the slides. After neutralisation, by washing in pH 7 buffer, the gels are stained with a DNA-binding dye and observed by fluorescence microscopy. A detailed protocol is available [5].

During electrophoresis, DNA, being negatively charged, is attracted to the anode, but it only moves appreciably if it contains breaks. A logical explanation of the formation of comets is based on the model of nuclear structure of Cook et al. [6]: DNA is attached at intervals to a nuclear

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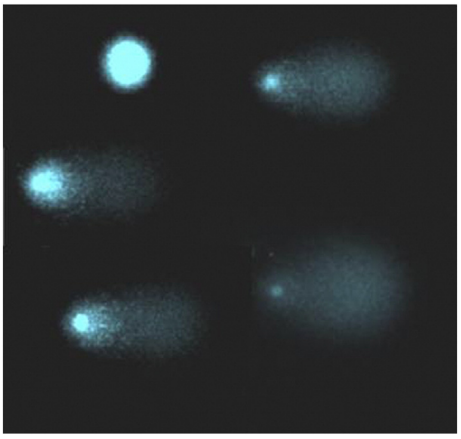

Fig. 1. Typical comet images from PBMN cells treated with H_2O_2, representing different levels of damage: top left, undamaged (no tail DNA); middle left, bottom left, top right — increasing levels of damage; bottom right — most damaged (almost all DNA in the tail). Comets were stained with 4′6-diamidine-2-phenylindol dihydrochloride (DAPI).

matrix and so is effectively a series of loops, which are the structural units. The DNA is supercoiled because it was wound around the histone cores of nucleosomes; although the histones are no longer present, the supercoiling remains because the DNA loops are constrained by their matrix attachment. A strand break relaxes supercoiling, and so broken loops are able to extend towards the anode, and it is these loops that form the comet tail. The relative size of the tail (most conveniently measured as the % of total fluorescence in the tail) reflects the number of DNA loops and therefore the frequency of DNA breaks.

2. Measuring oxidation damage to DNA

Reactive oxygen causes DNA breaks — but so do many other agents, and breaks can also appear as intermediates in DNA repair. A more specific indicator of oxidative attack is the presence of oxidised purines or pyrimidines. The basic comet assay was modified to detect these, by introducing an incubation of the nucleoids (just after lysis) with bacterial repair enzymes [7,8]. The enzymes combine a specific glycosylase activity, removing the damaged base and creating an apurinic/apyrimidinic (AP) site, and an AP lyase which converts the AP site to a break. Endonuclease III (Nth) is specific for oxidised pyrimidines, while formamidopyrimidine DNA glycosylase (FPG) acts on 8-oxo-7,8-dihydroguanine (8-oxoGua). The enzymes are available commercially, or can be isolated from over-producing strains of bacteria. An increase in % tail DNA after incubation with the enzyme, compared with an incubation with buffer alone, indicates the presence of oxidised bases (Fig. 2).

The enzyme-modified comet assay has been widely used, particularly in human biomonitoring, to determine background levels of oxidised bases in (usually) PBMN cells — commonly referred to as lymphocytes. It has been of particular interest to see whether the level of endogenous oxidative damage is affected by intervention with dietary antioxidants (or foods rich in antioxidants), and the many such studies have been reviewed [9,10]. The overall conclusion is that roughly half of the published studies show a decrease in base oxidation after supplementation, while the rest show no effect. Indications of an increase in damage are reassuringly rare. Whether a decrease in oxidised bases in the DNA of PBMN cells is of any significance for health is, however, an open question. It could be that a little bit of oxidative stress is good for us; immune reactions depend on ROS, and ROS have important roles in cell signalling [11]. However, increases in damage (including base oxidation) as a result of occupational or environmental exposure to genotoxins are likely to increase the risk of cancer, and the comet assay is a useful investigative tool in this area.

There have been reports of higher levels of oxidised bases associated with diabetes, cancer, arthritic, cardiovascular and neurodegenerative diseases (reviewed in [12]), but it is not clear whether the oxidative stress is a cause or a consequence of the disease. Studies of oxidation damage in relation to human aging have given mixed results. The recently established ComNet project [12] (www.comnetproject.org), a network of researchers using the comet assay as a human biomonitoring tool, has the aim of collecting data on DNA damage (and repair) in human subjects from as many studies as possible for pooled analysis. It is hoped that this will result in some firm conclusions about the role of DNA oxidation in human health, whether there are differences in damage levels between men and women, the relationship between oxidative damage and aging, and the influence of smoking and other lifestyle and environmental factors; it might also be possible to compare levels of damage in different countries.

3. Simple but not foolproof

The simplicity of the comet assay is deceptive. Care must be taken over practical details, and there are also some theoretical issues to consider.

Variations in the basic comet assay protocol can influence the results of an experiment quite profoundly. Two recent papers [13,14] independently identified the most critical factors. The first is the agarose concentration; the higher the concentration, the less DNA appears in the tail. Very low concentrations of agarose (below about 0.5%) are fragile, and the range of 0.6 to 0.8% is recommended. Obviously it is important to keep to the same concentration throughout a series of experiments. The density of comets is also important; there should not be so many that they overlap, because this makes scoring difficult, and if the density is too low, finding enough comets to score becomes a problem. We recommend placing a few thousand cells in a conventional large gel, or a few hundred in a mini-gel. All samples should be adjusted to a standard cell concentration, so that a fixed volume of cell suspension is added to a fixed volume of agarose to reach the required final agarose concentration (and cell density).

The period spent in lysis solution does not seem to matter. One hour is standard, but it is often extended to several hours or even days. However, the period of alkaline incubation prior to electrophoresis is important. Probably, the increase in breaks that occurs between 10 and 40 min in alkali (Fig. 3) is due to an increasing conversion of alkali-labile AP sites to frank breaks. Electrophoresis is the stage at which comets are created, and so it is not surprising that this seems to be the most critical stage. Varying the voltage gradient (measured across the platform carrying the slides) from 0.5 to 1.5 V/cm, or the electrophoresis time from 10 to 40 min, caused a proportional increase in % tail DNA. It is worth noting that in most publications the voltage quoted is the voltage shown on the power supply; the actual voltage gradient across the platform (which is rarely measured) will depend on the geometry of the tank and the depth of solution above the platform. The current does not affect comet formation, except indirectly, since an increase in the volume of solution in the tank will decrease the voltage gradient over the platform [14]. It is therefore good practice to keep to a standard volume of solution.

With the enzyme modification, there are of course additional factors to consider. Enzyme incubation conditions should be such that all relevant lesions are detected, without non-specific DNA breakage. Both enzyme concentration and incubation time need to be optimised, and this is done by a series of titration experiments with different concentrations of enzyme and different times. A substrate of cells containing the appropriate lesion is required. 8-OxoGua can be introduced into DNA by incubating cells with the photosensitiser Ro 19-8022 and irradiating with visible light [15], providing a suitable substrate for titrating FPG. For Nth, cells can be treated with H_2O_2 and incubated for an hour so that strand breaks are rejoined, leaving oxidised bases (which are only slowly repaired by the cells). Assuming that the supplier of the

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