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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 and biophysics of membrane proteins. Guest Editor: Christine Winterbourn.

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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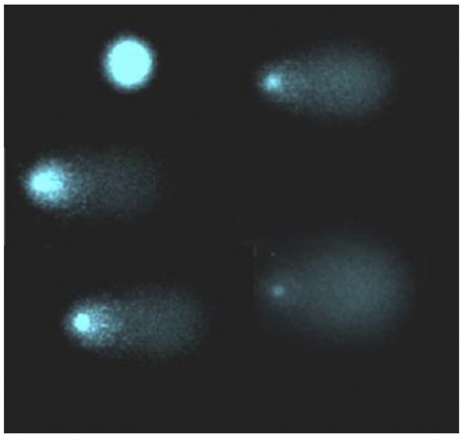

Fig. 1. Typical comet images from PBMN cells treated with H_2O_2, representing different levels of damage: top left, undamaged (no tail DNA); middle left, bottom left, top right — increasing levels of damage; bottom right — most damaged (almost all DNA in the tail). Comets were stained with 4′6-diamidine-2-phenylindol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

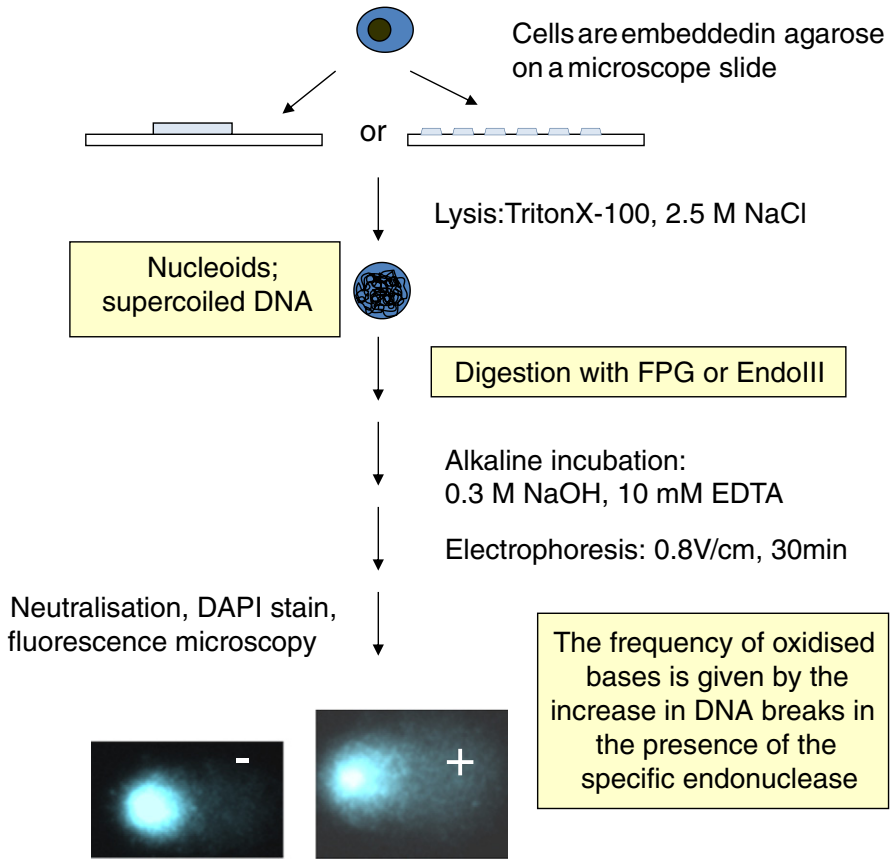

Fig. 2. Schematic representation of the comet assay with enzyme modification.

enzyme suggests a suitable working concentration, a series of dilutions is prepared around this concentration (1×, 3×, 10× higher and lower, for example) and 30 min incubations are carried out. A plateau should be reached at a certain concentration, indicating full lesion detection (if a crude enzyme preparation is used, non-specific nucleases present at low levels might lead to an increase in DNA breaks at high concentrations). If required, incubation time can be varied, with a fixed enzyme concentration; again, what is sought is a plateau level of breaks [13]. Enzyme solution is normally added to the gels while on ice. On transfer of slides to a 37 °C incubator, there will be a lag of several minutes as slides warm up before the reaction starts. The lag period should be identical in all experiments. The mini-gel system [3] is particularly convenient; a gasket separates each gel in its own well, so that different reagents or enzymes can be applied and all 12 gels are incubated under the same conditions. Smaller volumes are required than for standard gels, giving a cost saving.

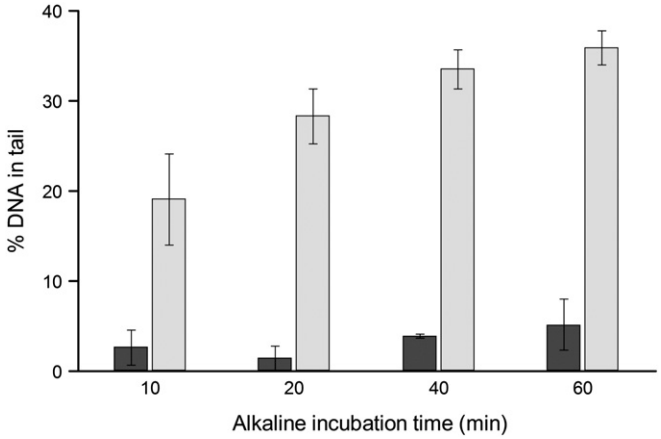

Fig. 3. DNA breaks increase with time in alkali before electrophoresis. TK-6 lymphoblastoid cells were untreated (dark shading) or incubated with 70 μM H_2O_2 (light shading). Mean values from two experiments are shown, the bars indicating the range of duplicates. From [14], with permission from Elsevier.

The dynamic range of the comet assay is up to about 3 breaks per 10^9 Da, with a limit of detection of about 0.1 break per 10^9 Da (this is determined by treating cells with different doses of ionising radiation; the yield of breaks per Gy is well established, at 0.3 per Gy per 10^9 Da [16]). Given a calibration curve, it is therefore possible to convert % tail DNA to breaks per 10^9 Da (or to breaks per cell, or breaks per million base pairs). Over the greater part of the dynamic range, the relationship between break frequency and % tail DNA is virtually linear, but above about 80% tail DNA, saturation is approached and the relationship departs from linearity. When estimating oxidised bases with the comet assay, it is usual to subtract the value of % tail DNA with buffer incubation, i.e. strand breaks/alkali-labile sites, from the % tail DNA with enzyme incubation to obtain 'net enzyme-sensitive sites'. This is reliable, unless the total damage (plus enzyme) approaches the saturation level, in which case simply subtracting the buffer score will lead to under-estimation of enzyme-sensitive sites. In this case, data should be converted to breaks per 10^9 Da first, using a calibration curve, and then the subtraction performed. As an example, assume that a particular treatment of cells leads to 32% tail DNA (above background) in buffer, and 60% tail DNA with enzyme. It seems that the amounts of strand breaks and altered bases are roughly equal. But — according to a typical calibration curve (from [17]) — 32% tail DNA is equivalent to 0.9 breaks per 10^9 Da, while 60% is equivalent to 2.5 breaks per 10^9 Da — implying that there are almost twice as many altered bases as strand breaks.

Although FPG is employed to measure 8-oxoGua, it is not a highly specific enzyme. As its name suggests, it was first identified as detecting formamidopyrimidines, i.e. ring-opened purines derived from damaged

(e.g. oxidised) adenine and guanine [18]. It was later found that a major substrate of this bacterial enzyme in vivo is 8-oxoGua [19] (since it has an associated AP-lyase activity, FPG will attack AP-sites too; but these lesions are alkali-labile, and so should be detected without enzyme incubation and therefore not included in the 'net enzyme-sensitive site' calculation). FPG also recognises some alkylation damage, namely N7-alkylguanines [20] or at least their ring-opened derivatives [19]. The mammalian counterpart of FPG is 8-oxoguanine DNA glycosylase (OGG). hOGG1, the human enzyme, has been cloned and characterised, and compared with FPG in a comet assay study; it is more specific than FPG, lacking activity against alkylated bases [21].

Whether enzyme specificity is something to worry about depends on the type of experiment. If it involves treating cells with an agent that is known to oxidise purines (such as Ro 19-8022 plus light), then FPG will give a reliable measure of effect. If, however, the aim is to measure endogenous DNA damage in human cells (as in biomonitoring studies), then it is important to know whether the damage is from oxidation or alkylation. Whether the background level of alkylated bases in normal human cells is significant compared with oxidised bases is not certain. In genotoxicity testing, if the aim is simply to know whether a compound has a genotoxic effect, then the less specific the enzyme is, the better; but to investigate the mechanism of action of a chemical, enzymes should be lesion-specific.

Ionising radiation causes clusters of lesions (2 or more closely spaced lesions), within cellular DNA, at a frequency four times higher than that of double strand breaks [22]. This could lead to underestimation of damage, since one break (or enzyme-sensitive site) per loop is sufficient to relax supercoiling and allow the DNA to enter the tail; clusters of lesions would therefore be counted as a single break. The occurrence of clustered lesions after treatment of cells with H_2O_2 is far rarer [23]. However, γ-irradiation of calf thymus DNA gave rise to a high proportion of tandem 8-oxoGua lesions [24], which — if replicated in cellular DNA with endogenous ROS — would imply an underestimation by the FPG-based methods.

4. Validating the enzyme-linked comet assay

Two aspects of validation will be discussed: first, reproducibility within and between laboratories; and second, comparisons between the comet assay and other methods for measuring 8-oxoGua in DNA.

In a typical biomonitoring study, there are so many samples that analysis requires many days of comet assay experiments. It is important to be able to trust and compare results, regardless of the day of the experiment, so it should now be standard practice to include reference standards in each experiment. These are samples of cells containing a known amount of damage. In the case of 8-oxoGua measurements, cells (e.g. PBMN cells) from a single batch are treated with Ro 19-8022 plus light, split into aliquots, and slowly frozen in aliquots to −80 °C where they are stored until needed. Negative controls (untreated cells) are prepared in parallel. Results (% tail DNA) from the reference standards should be constant (within the limits of experimental variation); a significant deviation indicates a problem with a particular experiment. What is an acceptable level of experimental variation within one laboratory? In a series of experiments including positive reference standards, the mean % tail DNA for net FPG-sensitive sites from 25 standard measurements was 47%, with a standard deviation of 6.7% — corresponding to a CV of 14% (unpublished results).

Given reference standard values, and assuming that whatever factors cause variation in these values will similarly influence the results for the samples under investigation, the latter can be adjusted appropriately, i.e. 'normalised' (a normalisation procedure is given in [25]).

The first serious comet assay 'ring study', i.e. comparison between laboratories, was carried out under the ESCODD (European Standards Committee for Oxidative DNA Damage) project [26]. HeLa cells were treated with Ro 19-8022 plus light in one lab and distributed (as frozen samples) to project partners in 11 different labs for analysis with FPG. Most labs used the comet assay to measure oxidised purines, but one used alkaline unwinding and another alkaline elution (two methods depending on the acceleration by strand breaks of the process of unwinding of DNA at high pH). The results ranged from 0.11 to 1.19 FPG-sites per 10^6 Gua, with a median of 0.53. In a later ESCODD trial [27], results were similarly diverse (Fig. 4). There is a hint of a consensus around the median value of 0.49 FPG-sites per 10^6 Gua, but the range, from 0.14 to 1.46 is not reassuring. However, as was illustrated by Møller et al. [28], the HeLa values could be used as a reference standard to 'correct' results with PBMN cells (collected in different laboratories); much of the dispersion seen in the original PBMN cell data then disappears — implying that there is little difference in background DNA oxidation in different European countries (Fig. 5). Since ESCODD came to an end, the task of organising ring studies has been taken over by ECVAG (European Comet Assay Validation Group) [29]. Participants were presented with samples treated with different concentrations of Ro 19-8022: 7 of 8 were able to detect the dose response in levels of FPG-sensitive sites, and the dose response slopes varied by a factor of slightly more than 2.

The ESCODD project was set up to examine the different ways of measuring 8-oxoGua — not just FPG-based methods, but also GC-MS and HPLC. It had become clear during the early 1990s that estimates of background levels of DNA oxidation using chromatographic methods tended to be an order or orders of magnitude higher than estimates using FPG [30]. Was FPG underestimating damage, or were the more analytical approaches overestimating it? There was some evidence already that during preparation for chromatography, oxidation of DNA could occur, and so one theme in ESCODD was to include antioxidants and free radical trapping agents at all possible stages. Identical samples of cells with different levels of oxidation of guanine were analysed in a ring study [31]. HPLC was used in 8 labs, and in 7 of these the slopes of the dose–response curve were essentially the same. However, the intercepts on the y-axis — representing the background level of damage in untreated cells — varied by a factor of almost a hundred. GC-MS and LC-MSMS were unable to detect the dose response. We concluded that chromatography was indeed seriously affected by adventitious oxidation of sample DNA and that this accounted for the higher estimates of background damage with these methods. Our efforts to eliminate the artefact by employing antioxidants, etc. were ultimately unsuccessful, though there have been improvements in the chromatographic methods in the past decade [32]. The FPG-based methods are apparently free of this artefact, presumably because the methods involved in sample preparation are much quicker and gentler, and so less likely to encourage oxidation.

It seems that, while HPLC is very precise at measuring induced oxidative damage, it is very inaccurate when estimating background levels. In contrast, FPG-based methods are imprecise (as is indicated by relatively high CVs when comparing labs), but probably more accurate. By analogy with archery, FPG results are centred on the bull's eye but scattered, while HPLC results are close together but some distance from the bull's eye.

5. Measuring antioxidant status

An early application of the comet assay was in investigating antioxidant status. When cells are treated with H_2O_2, breaks are induced, and the effectiveness of break production depends on the level of antioxidant defences within the cells. These defences include glutathione (a sulphydryl-rich tripeptide, present at high concentration, and of special importance in the nucleus) [33], the antioxidant enzymes catalase and superoxide dismutase, and micronutrients such as vitamin C, vitamin E, carotenoids, and various classes of polyphenolic compounds. Supplementation of volunteers with a mixture of vitamin C, vitamin E and β-carotene for 20 weeks resulted in a significant decrease in the yield of DNA breaks when PBMN cells were challenged ex vivo with H_2O_2 [34], and since then many studies involving supplementation for short

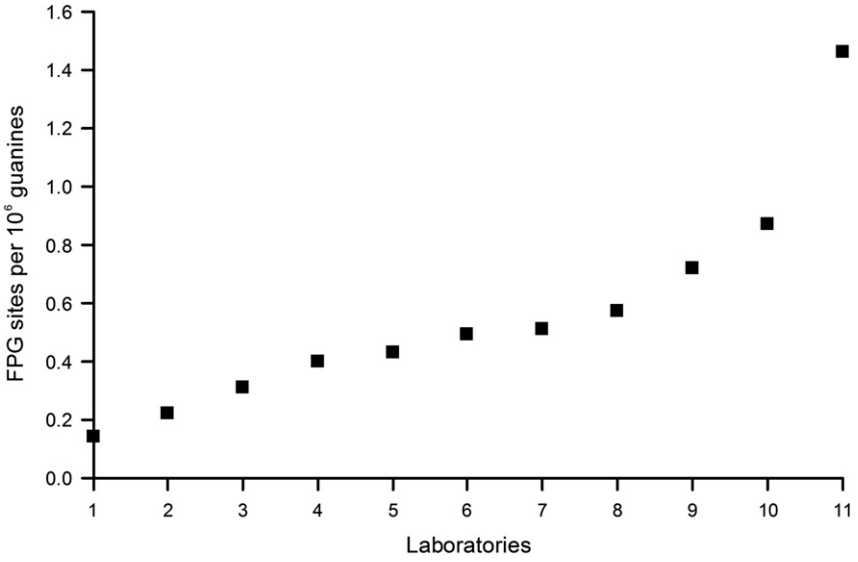

Fig. 4. Estimation of 8-oxoGua in HeLa cell DNA (treated with a single dose of Ro 19-8022 plus light) in different laboratories, arranged in increasing order of FPG-sensitive sites. Laboratory 8 used alkaline elution, and laboratories 6 and 10 alkaline unwinding; other laboratories used the comet assay.
Adapted from data in [27].

or longer periods, with a variety of micronutrient antioxidants or with plant foods, have shown similar improvements in antioxidant status [9].

6. Repair of oxidative damage to DNA, measured with the comet assay

If specific DNA lesions can be introduced into cellular DNA, then their removal by cellular repair processes can be monitored. Single strand breaks are typically repaired quickly, as is shown for proliferating HeLa and Caco2 cells in Fig. 6. If 8-oxoGua is induced by treating the cells with Ro 19-8022 and light, the repair pathway that deals with the damage is base excision repair, with the enzyme OGG1 carrying out the removal of the base and cleavage at the AP site (probably assisted by AP endonuclease). Removal of 8-oxoGua typically has a $t_{1/2}$ of a few hours (Fig. 6; [35]).

This cellular repair assay (also known as a challenge assay) is not ideal for measuring repair in samples collected in biomonitoring studies; it involves culturing cells for several hours, is best done on cells soon after collection, and it is not clear what is the best parameter for comparing repair rates between samples in which the initial levels of experimentally induced damage can vary widely — presumably reflecting differing antioxidant status [36].

An alternative approach was devised — an in vitro repair assay, in which a cell extract — usually from PBMN cells — is incubated with a substrate of agarose-embedded nucleoids containing a specific lesion (again, 8-oxoGua is most convenient if base excision repair of oxidative damage is to be monitored) [37]. Breaks accumulating with time of incubation are a measure of the repair capacity of the cell extract. We recently reviewed the use of this method in nutritional intervention trials, in studies of workers occupationally exposed to genotoxic chemicals, and in investigating the effect of repair gene polymorphisms [36]. The

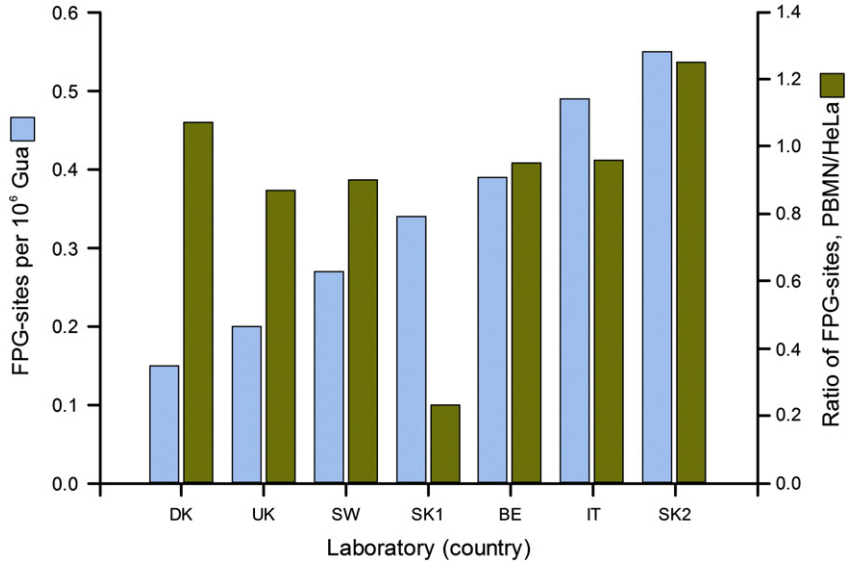

Fig. 5. Estimation of 8-oxoGua in DNA of PBMN cells in different countries. Cells were isolated from blood collected from between 8 and 20 healthy subjects in Denmark, United Kingdom, Sweden, Slovakia (2 laboratories), Belgium and Italy. Mean values of FPG-sites are shown as light blue bars. Each lab also measured FPG-sites in identical samples of untreated HeLa cells. The mean value of FPG-sites in PBMN cells in each lab was divided by the HeLa value for that lab, and the resulting ratios are shown as darker green bars (the lab 'SK1' had an anomalously high value for HeLa cells).
Adapted from data in [27].

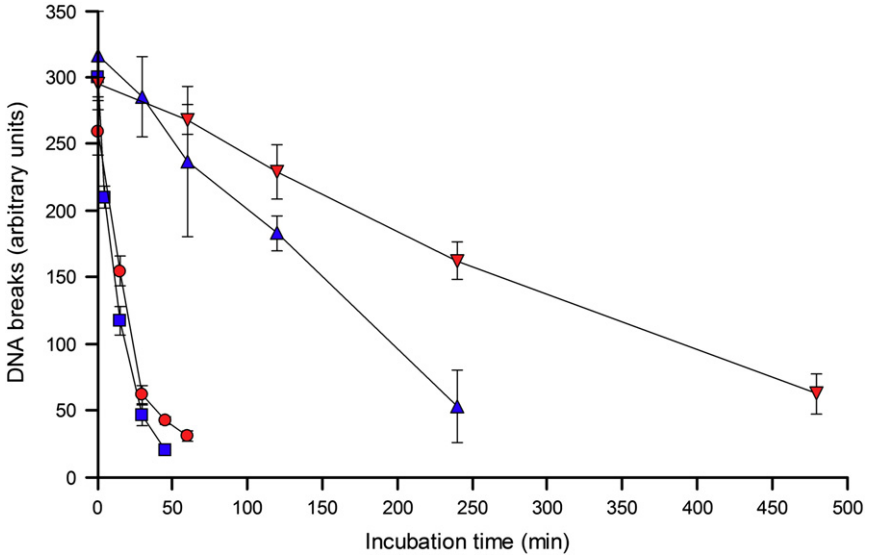

Fig. 6. Kinetics of rejoining of breaks induced by H_2O_2 (squares and circles) and repair of 8-oxoGua (triangles). Blue squares and triangles represent HeLa cells, and red circles and inverted triangles Caco2 cells. The cells were incubated at 37 °C. Mean values from three independent experiments are shown, with standard deviations (the background level of damage in these cells is around 15 arbitrary units).
Adapted from data in [35].

assay was recently adapted to compare repair capacities in tumour and normal tissue [38]. The relative contributions of genetic variation and environmental induction have yet to be resolved. A high intrinsic repair capacity might be expected to lead to a low steady state level of DNA damage and reduced risk of cancer; but on the other hand, a high repair rate might be a reflection of exposure to a high level of DNA-damaging agent.

7. Conclusions

The comet assay has a well-deserved popularity. It is simple and economical, requiring very little equipment (even a fluorescence microscope is not essential, as silver staining can be used [39]). It detects DNA damage in a 'physiological' range — i.e. at levels that are found in living cells treated with moderate doses of damaging agent. DNA breaks are produced by reactive oxygen species, but by other damaging agents too, and for more specific detection of oxidation damage, it is necessary to use enzymes that convert oxidised bases to breaks. This modification has been widely applied in human biomonitoring; it also has applications in ecogenotoxicology, and in genotoxicity testing to confirm the involvement of ROS.

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