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Single cell gel electrophoresis: Detection of DNA damage at different levels of sensitivity

Single cell gel electrophoresis, also known as the comet assay, is widely used for the detection and measurement of DNA strand breaks. With the addition of a step in which DNA is incubated with specific endonucleases recognising damaged bases, these lesions can be measured, too. In the standard protocol, electrophoresis is carried out at high pH. If, instead, electrophoresis is in neutral buffer, the effect of DNA damage seems to be much reduced – either because alkaline conditions are needed to reveal certain lesions, or because the effect of the same number of breaks on DNA migration is greater at high pH. A lower sensitivity can be useful in some circumstances, as it extends the range of DNA damage levels over which the assay can be used. Here we compare the performance of standard and modified techniques with a variety of DNA-damaging agents and offer possible explanations for the differences in behaviour of DNA under alternative electrophoretic conditions.

Keywords: Comet assay / DNA damage / Alkaline electrophoresis / Neutral electrophoresis
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1 Introduction

Single cell gel electrophoresis (the comet assay) is a sensitive method for measuring DNA strand breaks. After embedding cells in agarose on a microscope slide, they are lysed with Triton X-100 and 2.5 M NaCl to remove cytoplasm and most nuclear proteins, leaving supercoiled DNA as 'nucleoids'. On electrophoresis, DNA is attracted to the anode, but significant movement takes place only if breaks are present – in which case, a 'comet tail' of DNA extended from the nucleoid is seen. The percentage of DNA in this tail reflects the frequency of DNA breaks. One explanation for the behaviour of the DNA is that migration depends on the release of supercoiling (by strand breakage) so that the tail comprises relaxed loops of DNA. Comets can form under neutral as well as alkaline conditions of electrophoresis [1, 2]; however, the most commonly used variant of the method employs a period of treatment with NaOH/EDTA of pH above 13, prior to electrophoresis in this alkaline solution. Alkali allows unwinding of the double helix, and the presence of ssDNA in the comets is confirmed by the use of acridine orange to visualise them under fluorescence microscopy. While tails appear red (indicating ssDNA), the heads are yellow/green (typical of dsDNA) [2]. For whatever reason, alkaline conditions produce better-defined comets. The comets are easily analysed, by visual scoring (categorising comets into classes depending on the degree of damage) or by computer image analysis (in which case the % of DNA in the tail is a good indicator of damage).

A refinement of the assay involves digestion of DNA in the nucleoids with lesion-specific endonucleases. Endonuclease III, for instance, is specific for oxidised pyrimidines, revealing additional breaks at sites of base oxidation and therefore increasing comet tail intensity [3]. This modified comet assay has been particularly useful in biomonitoring studies, for instance investigating the importance of dietary antioxidants in protecting against oxidative DNA damage [4]. However, it was found that in the case of lymphocytes from workers exposed to DNA-damaging chemicals [5] or to radiation [6], the extra enzyme-detected sites may be impossible to measure accurately because, added to an already high level of strand breaks, they exceed the range of detection of the assay.

There is thus a need for a less sensitive version of the comet assay to extend its usefulness over a wider range of DNA damage levels. Recently, Koppen and Angelis [7] compared several procedures for measuring DNA breaks in nuclei from root cells of *Vicia faba*. Breaks were induced by different doses of X-irradiation. Under normal conditions (0.3 M NaOH/1 mM EDTA before and during electrophoresis), the dose response curve was linear only up to a dose of 10 Gy, corresponding to about 70% of DNA in the tail. Above that, the assay was clearly satu-

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Abbreviations: A/A, alkaline unwinding and alkaline electrophoresis; **A/N**, alkaline unwinding and neutral electrophoresis; **AP**, apurinic/apyrimidinic; **FPG**, formamidopyrimidine glycosylase; **MMS**, methylmethanesulphonate; **N/N**, neutral pre-incubation and neutral electrophoresis; **TBE**, Tris/borate/EDTA buffer

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rated, and increasing doses of X-rays had little effect. However, if the alkaline unwinding step (in NaOH/EDTA) was followed by electrophoresis in neutral TBE buffer (Tris/borate/EDTA), a linear dose response was maintained at least up to 50 Gy, with only 50% of DNA in the tail at that dose. There are two alternative explanations for the lower sensitivity of the modified assay. It is possible that some damage sites, seen as DNA breaks under alkaline electrophoretic conditions, remain unbroken when electrophoresis is performed at neutral pH; or there may be the same number of breaks present, but for some reason the breaks have different effects on the behaviour of DNA under the different conditions of electrophoresis.

We have now investigated the usefulness of this less sensitive comet assay in both animal and plant cells, with various different DNA-damaging agents and with a selection of lesion-specific enzymes to elucidate the basis for the difference in behaviour of DNA.

2 Materials and methods

2.1 Enzymes

Endonuclease III, formamidopyrimidine glycosylase (FPG) and T4 endonuclease V were prepared in the laboratory from over producing plasmid vectors. Minimal purification was necessary to remove nonspecific nuclease activities. They were used here at concentrations established as optimal for detection of their main substrates, *viz.* oxidised pyrimidines, 8-oxoguanine and cyclobutane pyrimidine dimers, respectively. Exonuclease III was obtained from Boehringer (Mannheim, Germany) and used at 100 U/mL under conditions recommended by the manufacturer.

2.2 Preparation of HeLa cells for comet assay

HeLa cells were grown in Nunclon plastic dishes (Life Technologies, Paisley, UK) in Eagle's Minimal Essential Medium with 5% foetal calf serum, 5% newborn calf serum, penicillin, and streptomycin. They were removed with trypsin, diluted with medium, centrifuged, and the pellet was suspended in PBS for treatment with methylmethanesulphonate (MMS; from Sigma, Poole, UK), menadione (Sigma) or Ro19-8022 (RO, a gift from Hoffmann-LaRoche, Basel, Switzerland). Menadione was present for 60 min at 37°C. MMS treatment was for 15 min at 37°C. Cells with RO were subsequently placed in a plastic dish on ice and irradiated with visible light from a 1000 W halogen lamp for 2 min. Treated cells were centrifuged, washed in PBS, centrifuged again and suspended in 1% low melting point agarose (Life Technologies) at a concentration of 2×10^5/mL. Two 85 μL aliquots of cells in agarose were placed on a glass microscope slide (precoated with 1% agarose and dried). Glass cover slips (22×22 mm) were placed on the gels, which were allowed to set at 4°C.

2.2.1 Alkaline unwinding/alkaline electrophoresis (A/A)

Cells embedded in agarose were lysed for 1 h in 2.5 M NaCl, 0.1 M Na_2EDTA, 10 mM Tris-HCl, pH 10, 1% Triton X-100 at 4°C. They were then washed three times with enzyme buffer (0.1 M KCl, 0.5 mM Na_2EDTA, 40 mM HEPES-KOH, 0.2 mg/mL BSA, pH 8.0) and incubated for 40 min at 37°C with endonuclease III, FPG or T4 endonuclease V in this buffer, or with buffer alone. Slides were then placed in 0.3 M NaOH, 1 mM Na_2EDTA (pH approximately 13) for 40 min before electrophoresis at 25 V (about 1 V/cm across the gels) and approximately 300 mA for 30 min at < 15°C. They were neutralised by dipping in 0.4 M Tris-HCl, pH 7.5, and then stained with 5 μg/mL of 4′,6-diamidino-2-phenylindole (DAPI). One hundred comets from each gel were analysed by fluorescence microscopy by visual inspection, giving each comet a value of 0–4 according to the degree of damage. The overall score was therefore between 0 and 400 arbitrary units. The score in arbitrary units correlates closely with the % DNA in the tail as estimated by computer image analysis, 300 units corresponding to approximately 55% of DNA in the tail [8].

2.2.2 Alkaline unwinding/neutral electrophoresis (A/N)

After incubation with buffer/enzyme and treatment with alkali, as described above, the slides were neutralised by dipping in 0.4 M Tris-HCl, pH 7.5, for 10 min and then transferred to TBE (45 mM Tris-borate, 1 mM EDTA, pH 8.0). They were electrophoresed for 10 min at 25 V, 10 mA at < 15°C. Staining and scoring were carried out as above.

2.3 Preparation of *Vicia faba* roots

Seeds of *Vicia faba* were soaked overnight in water and germinated at room temperature on moist Perlit beads. After 4–5 days, 1 cm of each growing root tip was removed. For each experimental point, five root tips were combined and kept in Hoagland solution (Sigma) on ice until used. They were treated with various concentrations of MMS in Hoagland solution, washed with PBS and finely chopped with a razor blade in 0.8 mL of PBS with 10 mM EDTA. This treatment released nuclei, which were cleaned of debris by filtering through a 20 μm nylon cloth. Forty μL of the nuclear suspension was mixed with

0.2 mL of 0.5% normal agarose (Life Technoligies) held liquid at 40°C and two 85 μL aliquots placed on a microscope slide previously coated with 0.5% agarose. A 22×22 mm glass cover slip was placed on each gel and the agarose was left to set. Embedded nuclei were lysed as described above for HeLa cells. Nuclear DNA was digested with exonuclease III for 1 h in 66 mM Tris-HCl, 0.66 mM $MgCl_2$, pH 7.5. Alkaline unwinding and then alkaline or neutral electrophoresis followed the same procedure as above. Comets stained with 5 μg/mL ethidium bromide were analysed by fluorescence microscopy, with the CometAs module of Lucia image analysis software (LIM; Praha, Czech Republic); the % DNA in the tail was measured for 100 comets per slide.

2.3.1 Neutral preincubation/neutral electrophoresis (N/N)

Neutral preincubation and neutral electrophoresis was adopted as a further variant; after enzyme treatment, slides were incubated for 10 min in TBE and then electrophoresed in TBE (10 min at 25 V, 10 mA).

3 Results and discussion

HeLa cells (transformed human endothelial cells) were treated with menadione, which is known to cause damage to DNA [9]. Figure 1 shows that, with human cells as with plant cells [7], for a given dose of damaging agent, the response of DNA under conditions of alkaline unwinding/neutral electrophoresis (A/N) is much less than under alkaline unwinding/alkaline electrophoresis (A/A). Under A/A conditions it is impossible to interpret the effect of incubating DNA with endonuclease III or FPG (which recognises altered purines, including the oxidation product 8-oxoguanine) after menadione treatment, because the level of DNA breakage is so high. In contrast, under A/N conditions it is apparent that these two enzymes do reveal significant additional damage site, *i.e.*, oxidised bases or apurinic/apyrimidinic (AP) sites (both endonuclease III and FPG have AP endonuclease activity as well as their specific glycosylase activity). This is in contrast to the report [9] that menadione induces – in rat hepatocytes – DNA strand breaks without base oxidation.

Oxidative damage is also introduced by visible light in the presence of the photosensitiser RO, but in this case it is mostly in the form of oxidised bases; the yield of strand breaks is relatively low [10, 11]. Under A/A conditions, these oxidised bases are revealed by the use of either endonuclease III or FPG (Fig. 2A), but the levels of (enzyme-induced) breaks are high enough to saturate the assay, and it is impossible to distinguish any dose response in terms of RO concentrations. A very different

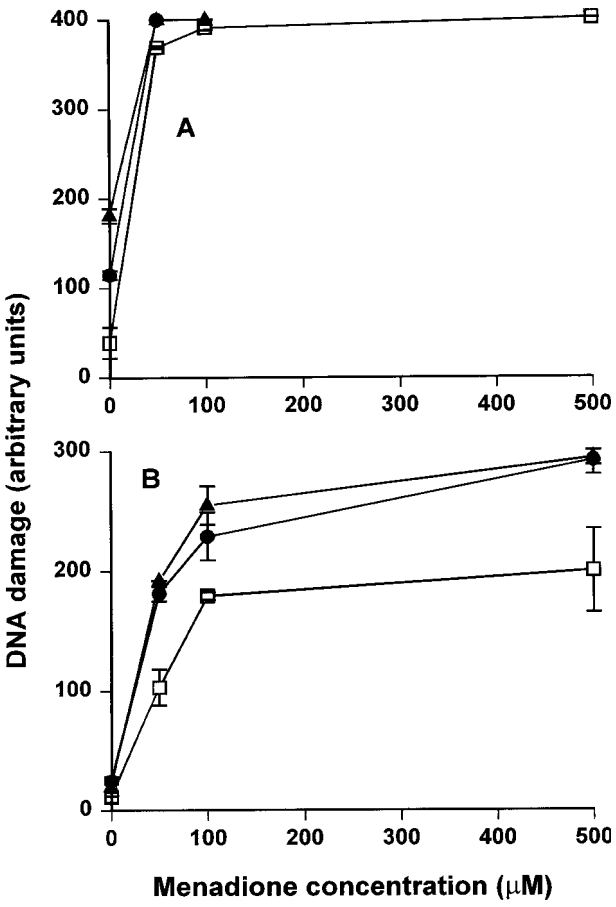

Figure 1. DNA damage induced by menadione in HeLa cells, detected with (A) alkaline unwinding and alkaline electrophoresis (conditions A/A) or (B) alkaline unwinding and neutral electrophoresis (conditions A/N). Strand breaks are indicated by open squares; incubation with FPG is represented by solid circles, and incubation with endonuclease III by solid triangles. Means are shown, with SE, or range of duplicate determinations.

picture is seen under A/N conditions (Fig. 2B); endonuclease III and FPG do not detect any base oxidation until an RO concentration of 0.75 μM is reached. T4 endonuclease V was also used in this experiment. This enzyme recognises cyclobutane pyrimidine dimers, induced by irradiation with UV light; it also has an associated AP endonuclease activity. AP sites are apparently not present in significant amount after treatment of cells with visible light/RO [10, 11]; the endonuclease V-dependent breaks thus most probably represent pyrimidine dimers. The yield is low compared with that of oxidised bases, as can be seen in Fig. 2B; under A/A conditions, however, with the assay saturated, there is no difference between effects of endonuclease V and the other two enzymes at any concentration of RO. In view of the absence of AP sites, the great difference between A/N and A/A results

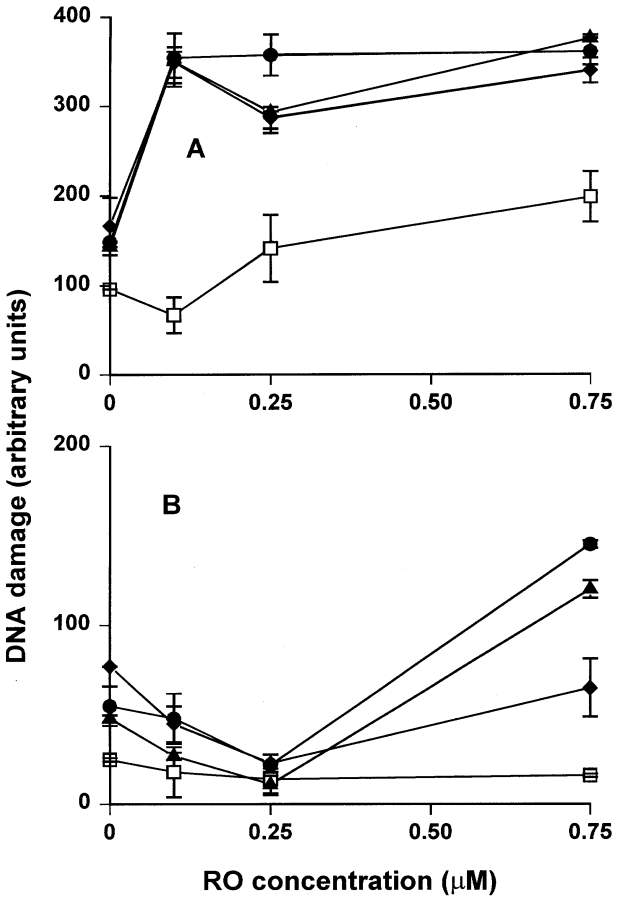

Figure 2. DNA damage induced in Hela cells by RO in combination with visible light, detected with (A) alkaline unwinding and alkaline electrophoresis (conditions A/A), or (B) alkaline unwinding and neutral electrophoresis (conditions A/N). Strand breaks are indicated by open squares; incubation with FPG is represented by solid circles, incubation with endonuclease III by solid triangles, and incubation with endonuclease V by solid diamonds. Means are shown, with SE, or range of duplicate determinations.

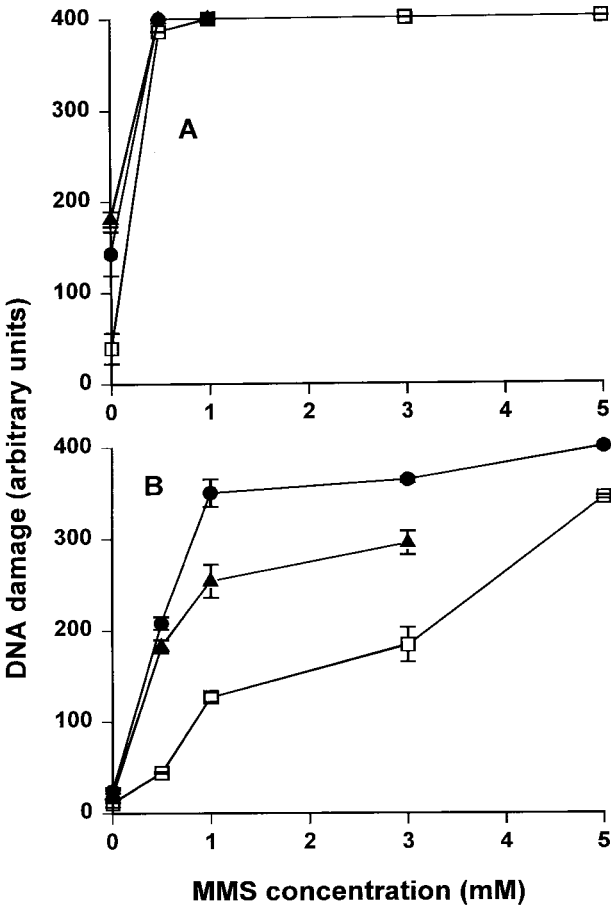

Figure 3. DNA damage induced in Hela cells by MMS, detected with (A) alkaline unwinding and alkaline electrophoresis (conditions A/A), or (B) alkaline unwinding and neutral electrophoresis (conditions A/N). Strand breaks are indicated by open squares; incubation with FPG is represented by solid circles, and incubation with endonuclease III by solid triangles. Means are shown, with SE, or range of duplicate determinations.

must lie in the different behaviour of damaged DNA under the two electrophoretic conditions – supporting the second of the hypotheses described above.

MMS was used to introduce alkylation damage in HeLa cell DNA (Fig. 3). The lowest dose of MMS used (0.5 mM) was sufficient to saturate the assay under A/A conditions. With A/N, a very clear picture is seen, with strand breaks and enzyme sites increasing with MMS treatment. In this case, while endonuclease III is presumably detecting AP sites (as MMS does not induce oxidative damage), FPG may also recognise ring-opened purines which are likely to result from alkylation damage [12]. It is apparent that at least some AP sites – although they are alkali-labile – are not susceptible to conversion to breaks under A/N conditions. However, from these results nothing can be deduced about the extent of conversion of AP sites to breaks under A/A conditions.

Figure 4 illustrates experiments carried out on *Vicia faba* root tip cells, treated with MMS or menadione. As with HeLa cells, the effects of MMS (especially at the higher concentration) were greater – in terms of comet formation – under A/A than under A/N conditions (Fig. 4A). Also shown in this figure are the results obtained with electrophoresis in TBE buffer without prior alkaline treatment (N/N). Although the background level of DNA in the tail is rather high, the increase seen in MMS-treated cells is very small. The DNA tail seen in untreated cells under N/N conditions, clearly not the result of extra DNA break-

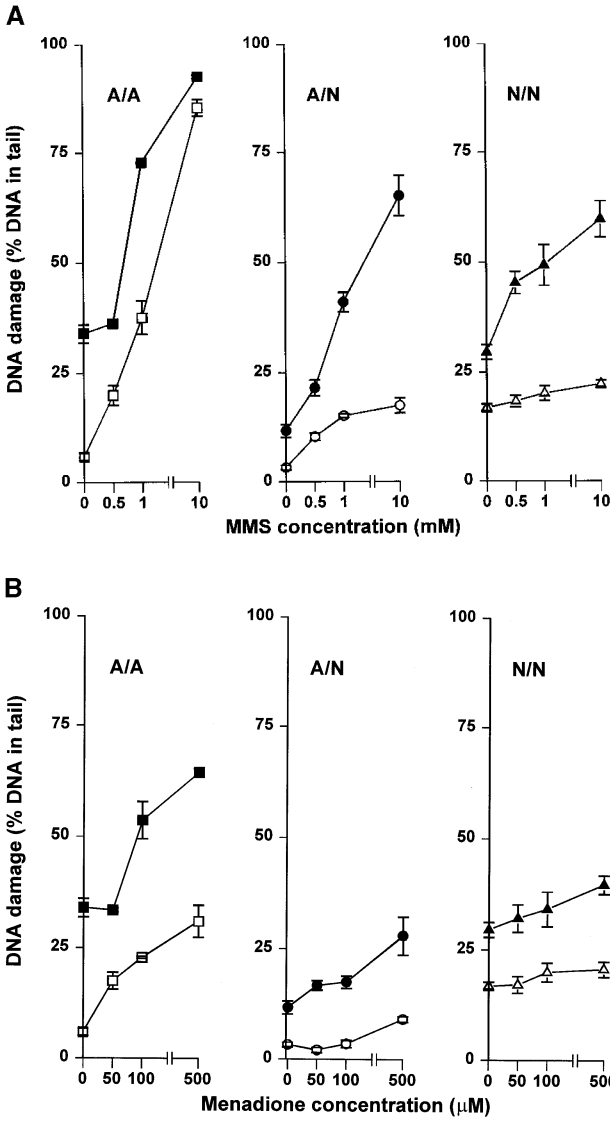

Figure 4. DNA damage induced in *Vicia faba* root tip cells by (A) MMS, (B) menadione, detected with alkaline unwinding and alkaline electrophoresis (conditions A/A), alkaline unwinding and neutral electrophoresis (conditions A/N), or neutral preincubation and neutral electrophoresis (conditions N/N). Strand breaks are indicated by open symbols; incubation with exonuclease III is represented by solid symbols. Mean (with SE) is shown (n=6).

age, may instead reflect a distortion of the shape of the nucleoid under electrophoresis, associated with the fact that the DNA is not denatured as it is under alkaline conditions. Incubation with the enzyme exonuclease III – an AP endonuclease – was included in the tests of all three conditions; a substantial increase in breaks was seen as the concentration of MMS increased. Again, therefore, AP sites are resistant to A/N conditions. There are also AP endonuclease-sensitive sites resistant to A/A conditions; the enhanced comet tail intensity in the presence of enzyme is striking, though many of the sites are present in untreated cells.

The damage caused by menadione in the plant cells (Fig. 4B) is not as great as in HeLa cells at comparable concentrations (compare Fig. 1), and A/A conditions in this case are optimal for detection of damage. Menadione seems not to induce significant numbers of AP endonuclease-sensitive sites, judging by the relatively slight divergence of the curves (with and without AP endonuclease) in all three conditions.

4 Concluding remarks

Overall, it seems likely that the lower sensitivity of the comet assay with A/N compared with A/A conditions is due to the different behaviour of DNA on electrophoresis. It is clear that A/N conditions do not detect AP sites (at least, not all AP sites), and the same appears to be true for A/A conditions in spite of the longer exposure to alkali. The modified comet assay with A/N conditions gives consistent results, shows good dose responses for several DNA-damaging agents, and should prove valuable for estimating damage over a wider range than is possible with the conventional (A/A) comet assay. It tends to give a lower background level of comet tail intensity than the all-neutral (N/N) assay.

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