TECHNICAL NOTE

In vitro comet assay for DNA repair: a warning concerning application to cultured cells

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The comet assay (single-cell gel electrophoresis) is a sensitive and simple method for measuring DNA damage. An early modification of the assay involved the application of specific repair endonucleases to convert lesions to breaks; thus, for example, endonuclease III was used to measure oxidized pyrimidines (1). This concept has now been extended to produce an in vitro assay for DNA repair activity in a cell-free extract, for example from lymphocytes. The extract is incubated with substrate DNA containing specific base damage, and repair incision is detected as breaks in this DNA. We have recently been studying effects of phytochemicals in cultured cells, whether as antioxidants or as potential modulators of DNA repair. We realized that there is a need to check that observed effects that appear as an enhancement of repair (i.e. increased breaks in substrate DNA) are not simply due to a direct damaging effect of the phytochemical or to induction of non-specific nucleases. Here, we describe a rigorous approach to testing for this possibility, which we recommend to anyone carrying out similar experiments.

The comet assay (single-cell gel electrophoresis) is a sensitive and simple method for measuring DNA damage. The presence of breaks in the DNA relaxes supercoils which then extend under electrophoresis to form the characteristic image of a comet tail. An early modification of the assay involved the application of specific repair endonucleases to convert lesions to breaks; thus, for example, endonuclease III was used to measure oxidized pyrimidines (1). This concept has now been extended to produce an in vitro assay for DNA repair activity in a cell-free extract, for example from lymphocytes (2). The substrate for the assay consists of cells treated with a specific DNA-damaging agent and then embedded in agarose on a microscope slide and lysed to produce nucleoids, as in the standard comet assay. The cell-free extract is incubated with this substrate and the breaks introduced in the nucleoid DNA represent the repair activity of the extract. If the substrate cells were treated with the photosensitizer Ro 19-8022 + visible light, inducing 8-oxoG in the DNA, then the assay measures incisions by 8-oxoG DNA glycosylase (OGG). This first version of the assay measuring base excision repair (BER) has been employed in human biomonitoring to check for effects of occupational or experimental exposure to various agents (3–7), in nutritional intervention studies (8–10) and in studies of effects of polymorphisms in genes for DNA repair on the DNA repair phenotype (11,12). A modified version, to measure nucleotide excision repair, is based on substrate cells treated with benzo[a]pyrene (13) or with ultraviolet light, UV(C) (14).

We have recently been studying effects of phytochemicals in cultured cells, whether as antioxidants or as potential modulators of BER (15). Typically, cells are preincubated with the phytochemical, the cell-free extract then prepared and incubated with the substrate DNA containing 8-oxoG. In this paper, we draw attention to a potential problem facing users of the in vitro repair assay with cultured cells and specify procedures to avoid it. We encountered complications while investigating the effect of β-cryptoxanthin or vitamin C on BER. The incision activity of extracts from HeLa cells treated with non-genotoxic doses of β-cryptoxanthin or vitamin C was measured by incubating the extracts with DNA substrate nucleoids containing 8-oxoG. In both cases, extracts from treated cells showed a significant increase in breaks compared with extracts from non-treated cells, so we concluded that these extracts contained a higher activity of OGG (Figures 1 and 2). Nevertheless, to exclude the possibility of a direct effect of antioxidants on DNA, we treated the nucleoids with low concentrations of β-cryptoxanthin (between 0.1 and 4 μM) or vitamin C (0.25–6.25 μM). In fact, breaks were present after incubation of DNA with vitamin C, and so the apparent increase in the incision activity of extracts from HeLa cells treated with vitamin C probably did not represent OGG activity but could simply be a direct effect of this antioxidant on DNA (Figure 1). However, β-cryptoxanthin did not reveal any such direct effect on DNA (data not shown).

There is another possible explanation of the apparent enhancement of repair incision by β-cryptoxanthin—that it is stimulating non-specific nuclease activity. To check this possibility, extract from HeLa cells treated with β-cryptoxanthin was incubated with non-damaged nucleoids. Figure 2 demonstrates that there was no increase in breaks above the basal level; therefore, the increase in DNA breaks seen with extract from β-cryptoxanthin-treated cells must indicate a real increase in repair activity.

The relevant concentrations to test for a direct effect will depend on how well the compound is taken up into the cell and on its metabolism within the cell and will therefore vary from compound to compound and from cell line to cell line. Vitamin C is unstable in cell culture medium [40% depletion after 2-h incubation (16)] and in addition it is not known whether HeLa cells retain the active transport mechanisms responsible in normal cells for uptake of vitamin C (17); therefore, it seemed reasonable to test concentrations of 0.25, 1.25 and 6.25 μM vitamin C (substantially lower than the 200 μM initially present in the medium). Since effects were seen at such low concentrations, it was not necessary to explore higher concentrations. In contrast, the uptake and metabolism of β-cryptoxanthin are...
unknown and so we applied concentrations in the same range as were present in the medium and found no effect.

As these results illustrate, an increase in breaks caused by the extract from cells treated with some test compound in the in vitro repair assay does not necessarily mean that the compound is inducing an increase in OGG activity. It may also be due to a direct effect of the test compound or to an increase in the activity of non-specific nucleases. Appropriate control experiments should always be performed, as illustrated schematically in Figure 3.

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**References**


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**Fig. 1.** Effect of (A) extracts from HeLa cells pre-treated with 0 or 200 µM vitamin C for 6 h and (B) 0.25, 1.25 or 6.25 µM vitamin C solutions in buffer, incubated with DNA substrate containing 8-oxoG for 20 min. Controls incubated with buffer or FPG are also shown. Standard deviation values based on the results of three independent experiments. *P* ≤ 0.05, comparing (A) extracts with and without vitamin C preincubation and (B) presence or absence of vitamin C.

**Fig. 2.** Incision by HeLa extracts incubated for 10 min with gel-embedded nucleoid DNA from cells treated with Ro 19-8022 plus light to induce 8-oxoG (black bars) or nucleoids from untreated cells (white bars). HeLa cells were incubated with β-cryptoxanthin (0, 1 and 4 µM as indicated) for 2 h before preparing extracts. Controls incubated with buffer or formamidopyrimidine DNA glycosylase (FPG) are also shown. Standard deviation values based on the results of three independent experiments are shown. *P* ≤ 0.05, comparing extracts with and without β-cryptoxanthin preincubation. Redrawn from ref. (13).

**Fig. 3.** Different stages in the DNA repair assay indicating where tests are performed.


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