



Editorial

Introduction to hCOMET special issue, 'Comet assay *in vitro*'



This special issue is produced as a valuable outcome of the COST Action hCOMET. A major aim of this project is to encourage the adoption of standard procedures for the comet assay, in order to improve reliability. Comet assay procedures tend to vary from one laboratory to another, and so comparing results between laboratories can be problematic. Much work has been done to establish how different parameters, such as agarose concentration, lysis time, or electrophoresis voltage gradient can affect results, and an aspiration of hCOMET is to devise standard procedures. In addition to papers on practical issues relating to the use of the comet assay, there are several descriptions of novel model cell systems. Finally, there are accounts of various applications of the assay to *in vitro* genotoxicity testing (mainly on human cell lines), as well as investigations of antigenotoxicity.

1. Methods

Intra- and inter-experimental variability continues to be a concern for comet assay researchers. A sure way to control variability is to include reference standards (i.e. cells with known amounts and types of DNA damage) in all experiments, so that results from samples can be adjusted according to variation in the standard. Internal reference standards - i.e. reference cells that can be distinguished from sample cells and so can be included in the same gel - are the ideal solution, and are being developed (Brunborg [1]).

Harvesting of cultured cells, and frozen storage, are procedures common to many laboratories, and it is likely that each laboratory has its own preferred technique for each procedure. However, a systematic comparison of methods is rare. Bessa et al. [2] compared, in two human cell lines, harvesting by scraping and enzymic detachment, and freezing in either serum or culture medium (with 10% DMSO in each case). On the whole, enzymic detachment and culture medium gave better results (i.e. lower levels of DNA breaks), while freezing itself had no detrimental effect.

The enzyme-modified comet assay allows the determination of specific DNA lesions. Formamidopyrimidine DNA glycosylase (Fpg) is the most often used enzyme, detecting oxidised purines. Optimisation of the reaction conditions is done by titration, varying enzyme concentration and incubation time; the aim is to detect the maximum number of enzyme-sensitive sites without inducing non-specific DNA breakage. Muruzabal et al. [3] describe titration experiments with the standard set-up of 2 gels per slide, and the 12-mini-gels-per-slide system in which each gel is contained in a well containing a fixed volume of enzyme. A 10x lower concentration of Fpg was optimal for mini-gels compared with 2 gels per slide.

Mycoplasma infection is a cause for serious concern in cell culture laboratories; it is difficult to detect or treat, and leads to anomalous cell behaviour. Among its detrimental effects, as shown by Ji et al. [4], are

the induction of DNA damage – both strand breaks and oxidised bases (detected with hOGG1, the human counterpart of Fpg) – and a serious delay in the repair of oxidised bases.

2. Model cell systems

Clearly human tissues differ in cellular physiology, metabolic activity and therefore also in their susceptibility to xenobiotics. The use of a 'generic' cell line, such as HeLa, can give misleading results. There is therefore a quest for tissue-specific cell lines and culture systems suitable for genotoxicity testing.

Reviewing 105 papers that describe genotoxicity testing in human colon cells, Bankoglu et al. [5] found that HT29, Caco2 and HCT116, established from colorectal adenocarcinomas, were the most frequently used cancer cell lines; there were 5 reports of primary cultures from normal colon, and 2 of colon cancer biopsies. They express reservations about the use of tumour-derived cells when investigating genotoxic agents and cancer risk (since normal cells are the *in vivo* target), but they are appropriate for studies in the area of colon cancer therapy.

The paper by Misik et al. [6] is a comprehensive review of the various liver-derived cell lines that have been used in genotoxicity testing. The important feature of liver cells is their complement of phase I and phase II metabolising enzymes; in theory, they should allow a realistic assessment of the DNA-damaging and mutagenic effects of chemicals, including those that act indirectly (i.e. that require activation by the liver enzymes). Cell lines, however, are never as effective at xenobiotic metabolism as are primary liver cells. 3D cell systems are recognised as being more realistic tissue models, compared with conventional 2D cell culture, and Elje et al. [7] examined whether spheroids created in hanging drops of the liver derived cell line HepG2 might be an appropriate model for genotoxicity testing. H₂O₂ treatment of (disaggregated) spheroids and 2D cultures gave similar levels of strand breaks, and after incubation of 2D cultures or spheroids with methyl methane sulphonate for 24 h, strand breaks and altered purines were detected with similar concentration dependence in both. It remains to be seen whether the spheroids are as effective at detecting indirect-acting genotoxins.

The use of neural cell lines in genotoxicity testing was reviewed by Kruszewski et al. [8], concentrating on environmentally or occupationally relevant agents such as fire retardants, plant protection agents (insecticides), nanoparticles and magnetic field. The authors noted the wide variation in comet assay methods, which makes inter-laboratory comparisons dubious and reviews challenging. Neuronal and glial cells were used by Fernandez-Bertolez et al. [14] to test nanoparticles, as described below.

Human kidney model cell systems are reviewed by Gabelova et al. [9] They, too, observed the lack of a standard comet assay procedure

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and the difficulties this poses. Almost 50 chemicals, as well as various nanomaterials, have been studied using three human kidney cell lines. The human renal proximal tubule epithelial (TH1) cell line is promising, as it retains renal functions and shows normal, contact-inhibited, anchorage-dependent growth. Sramkova et al. [10] used TH1 cells as a model system for testing cytotoxicity and genotoxicity of titanium dioxide, silica, magnetite and gold in nanoparticle form. They were taken up by the cells to varying extents, and none induced either DNA strand breaks or oxidised bases.

Montag et al. [11] were concerned not with cell/tissue type, but with the state of differentiation of cells – specifically, human promyelocytic HL60 cells which can be induced to differentiate to granulocyte-like cells on treatment with DMSO. Tested with H₂O₂ or KBrO₃, differentiated and undifferentiated cells showed similar levels of DNA damage. With methyl methane sulphonate, damage was slightly greater in undifferentiated cells; etoposide caused at least twice as many strand breaks in undifferentiated compared with differentiated cells, while doxorubicin caused breaks in undifferentiated but no damage in differentiated cells. The state of differentiation is likely to affect the response of other cells types – in vivo as well as in vitro.

3. Genotoxicity and antigenotoxicity testing

Genotoxicity can result from exposure to physical as well as chemical agent. Brech et al. [12] studied effects of intermediate frequency magnetic fields on canine and human blood cells. Frequencies selected are common in medical, industrial and household devices. Exposure for 20 h caused significant increases in strand breaks. Use of a specially designed Petri dish led to the conclusion that the magnetic flux, rather than an induced electric field, caused the damage.

Diesel and biodiesel fuels produce particulate matter which contains known carcinogens. In a preliminary study, Novotna et al. [13] tested extracts from the exhaust of engines simulating different traffic conditions; both fuels induced DNA damage (mostly strand breaks, with some oxidised bases) after 4 h exposure; yet at 24 h, in the case of biodiesel, damage was at or near control levels. Therefore biodiesel seems to be less genotoxic than regular diesel fuel, perhaps reflecting the lower concentrations of polycyclic aromatic hydrocarbons in the former.

The list of nanomaterials, and their applications, is expanding with no end in sight; yet there is growing evidence of (geno)toxicity resulting from their small size, increased reactivity and ability to penetrate cells. The challenge of testing all nanomaterials is compounded by the fact that different coatings can confer different properties, and reactivity can also vary with the tissue, or cell type tested. Fernandez-Bertolez et al. [14] tested nanoparticles of magnetite (iron oxide) coated with either silica or oleic acid, in A172 human glioblastoma cells and SH-SY5Y neuroblastoma cells. Using the comet assay together with OGG1, they showed that both nanomaterials induced DNA base oxidation (but not strand breaks) in both cell types. TiO₂ is approved as a food additive (E171) and is also very common in the form of nanoparticles. Using a model for the human intestine, comprising co-culture of Caco2 and HT29-MTX cells, Doner et al. [15] reassuringly found no significant induction of strand breaks or oxidised purines by either form of TiO₂.

Opattova et al. [16] report an apparently selective genotoxic effect of an extract of *Ganoderma lucidum*, a mushroom used in traditional eastern medicine; oxidised DNA bases were induced in two cancer-derived cell lines but not in a non-malignant cell line. The extract also sensitised the cancer cells to the DNA-damaging and anti-proliferative effects of the chemotherapy drug 5-fluorouracil.

'Immune Assist' is a complex mixture of polysaccharides and other water-soluble compounds prepared from 6 species of medicinal mushrooms. It was tested by Živković et al. [17] for its ability to block the oxidative effect of hydrogen peroxide on human peripheral blood cells. Cells incubated with Immune Assist before treatment with H₂O₂ showed markedly reduced levels of DNA strand breaks; and if Immune Assist was present after H₂O₂ treatment, removal of strand breaks was

accelerated. Thus Immune Assist appears to be both an antioxidant and a stimulator of DNA repair. A similar approach was used by Topalović et al. [18] to investigate potential protection by an extract of olive leaves against DNA-damaging effects of 17β-estradiol and diethylstilbestrol, with similar results, though the effects are ascribed to an enhancement of cellular antioxidant defences and direct scavenging of free radicals.

Gerčić et al. [19] explored the radioprotective effect of sodium copper chlorophyllin – a permitted food colorant – incubated with human whole blood before γ-irradiation. The yield of strand breaks was significantly reduced. As the compound is non-cytotoxic, it might have applications in radiotherapy (protecting non-target tissue), perhaps presented in the form of nanoparticles. The protective effects of another compound, AV-153 1,4-dihydropyridine, with antioxidant and anti-mutagenic properties, were found to be modified to varying extents by the presence of different metal ions in the salts of the compound (Leonova et al. [20]).

While hCOMET as a project is primarily concerned with human biomonitoring, other aspects of the comet assay also engage members of the COST Action. This range of interests is reflected in this collection of articles. The two hCOMET special issues are truly complementary.

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