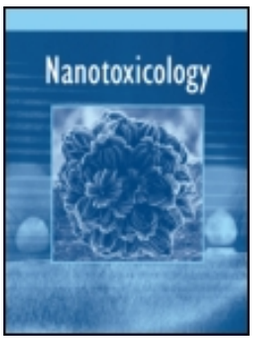

Nanotoxicology

ISSN: 1743-5390 (Print) 1743-5404 (Online) Journal homepage: https://www.tandfonline.com/loi/inan20

Suitability of human and mammalian cells of different origin for the assessment of genotoxicity of metal and polymeric engineered nanoparticles

Hilary Cowie, Zuzana Magdolenova, Margaret Saunders, Martina Drlickova, Sara Correia Carreira, Blanka Halamoda Kenzaoi, Lourdes Gombau, Rina Guadagnini, Yolanda Lorenzo, Laura Walker, Lise Marie Fjellsbø, Anna Huk, Alessandra Rinna, Lang Tran, Katarina Volkovova, Sonja Boland, Lucienne Juillerat-Jeanneret, Francelyne Marano, Andrew R. Collins & Maria Dusinska

To cite this article: Hilary Cowie, Zuzana Magdolenova, Margaret Saunders, Martina Drlickova, Sara Correia Carreira, Blanka Halamoda Kenzaoi, Lourdes Gombau, Rina Guadagnini, Yolanda Lorenzo, Laura Walker, Lise Marie Fjellsbø, Anna Huk, Alessandra Rinna, Lang Tran, Katarina Volkovova, Sonja Boland, Lucienne Juillerat-Jeanneret, Francelyne Marano, Andrew R. Collins & Maria Dusinska (2015) Suitability of human and mammalian cells of different origin for the assessment of genotoxicity of metal and polymeric engineered nanoparticles, Nanotoxicology, 9:sup1, 57-65, DOI: 10.3109/17435390.2014.940407

To link to this article: https://doi.org/10.3109/17435390.2014.940407

http://informahealthcare.com/nan
ISSN: 1743-5390 (print), 1743-5404 (electronic)

Nanotoxicology

Nanotoxicology, 2015; 9(S1): 57–65
 DOI: 10.3109/17435390.2014.940407

informa
healthcare

ORIGINAL ARTICLE

Suitability of human and mammalian cells of different origin for the assessment of genotoxicity of metal and polymeric engineered nanoparticles

Hilary Cowie[1], Zuzana Magdolenova[2], Margaret Saunders[3], Martina Drlickova[4], Sara Correia Carreira[5], Blanka Halamoda Kenzaoi[6,7], Lourdes Gombau[8], Rina Guadagnini[9], Yolanda Lorenzo[10], Laura Walker[11], Lise Marie Fjellsbø[2], Anna Huk[2], Alessandra Rinna[2], Lang Tran[1], Katarina Volkovova[12], Sonja Boland[9], Lucienne Juillerat-Jeanneret[6], Francelyne Marano[9], Andrew R. Collins[10], and Maria Dusinska[2]

[1]Institute of Occupational Medicine, Research Avenue North, Riccarton, Edinburgh, UK, [2]Health Effects Laboratory MILK, NILU – Norwegian Institute for Air Research, Kjeller, Norway, [3]BIRCH, Department of Medical Physics & Bioengineering, St Michael's Hospital, University Hospitals Bristol NHS Foundation Trust, Bristol, UK, [4]Centre for Chemical Substances and Preparations, Bratislava, Slovakia, [5]Bristol Centre for Functional Nanomaterials, University of Bristol, Bristol, UK, [6]University Institute of Pathology, CHUV-UNIL rue du Bugnon 25, Lausanne, Switzerland, [7]European Commission, Joint Research Centre, NanoBioSciences Unit, Ispra (VA), Italy, [8]LEITAT Technological Center, Barcelona, Spain, [9]Univ Paris Diderot, Sorbonne Paris Cité, Unit of Functional and Adaptive Biology (BFA), Laboratory of Molecular and Cellular Responses to Xenobiotics (RMCX), Paris, Cedex, France, [10]Department of Nutrition, University of Oslo, Blindern, Oslo, Norway, [11]Bristol Heart Institute, School of Clinical Sciences, University of Bristol, Bristol, UK, and [12]Faculty of Medicine, Slovak Medical University, Limbova, Bratislava, Slovakia

Abstract

Nanogenotoxicity is a crucial endpoint in safety testing of nanomaterials as it addresses potential mutagenicity, which has implications for risks of both genetic disease and carcinogenesis. Within the NanoTEST project, we investigated the genotoxic potential of well-characterised nanoparticles (NPs): titanium dioxide (TiO_2) NPs of nominal size 20 nm, iron oxide (8 nm) both uncoated (U-Fe_3O_4) and oleic acid coated (OC-Fe_3O_4), rhodamine-labelled amorphous silica 25 (Fl-25 SiO_2) and 50 nm (Fl-50 SiO) and polylactic glycolic acid polyethylene oxide polymeric NPs – as well as Endorem® as a negative control for detection of strand breaks and oxidised DNA lesions with the alkaline comet assay. Using primary cells and cell lines derived from blood (human lymphocytes and lymphoblastoid TK6 cells), vascular/central nervous system (human endothelial human cerebral endothelial cells), liver (rat hepatocytes and Kupffer cells), kidney (monkey Cos-1 and human HEK293 cells), lung (human bronchial 16HBE14o cells) and placenta (human BeWo b30), we were interested in which *in vitro* cell model is sufficient to detect positive (genotoxic) and negative (non-genotoxic) responses. All *in vitro* studies were harmonized, i.e. NPs from the same batch, and identical dispersion protocols (for TiO_2 NPs, two dispersions were used), exposure time, concentration range, culture conditions and time-courses were used. The results from the statistical evaluation show that OC-Fe_3O_4 and TiO_2 NPs are genotoxic in the experimental conditions used. When all NPs were included in the analysis, no differences were seen among cell lines – demonstrating the usefulness of the assay in all cells to identify genotoxic and non-genotoxic NPs. The TK6 cells, human lymphocytes, BeWo b30 and kidney cells seem to be the most reliable for detecting a dose-response.

Keywords

Cell models, comet assay, genotoxicity, nanoparticles, statistical analysis

History

Received 28 February 2014
Revised 1 June 2014
Accepted 26 June 2014
Published online 19 August 2014

Introduction

Within the EC FP7 NanoTEST project (alternative testing strategies for the assessment of the toxicological profile of nanoparticles (NPs) used in medical diagnostics), a range of medically relevant engineered NPs have been evaluated (Juillerat et al., 2015) to determine whether or not they can cause toxicity in a broad range of cell lines and primary cells and if so, at what exposure levels. This article compares NP genotoxicity across a range of primary cells/cell lines as determined by the comet assay.

DNA damage is a critical molecular event that can result in mutation and consequently lead to the development of cancer and other diseases. If the genetic event occurs in germ cells, it may cause genetic disease or reproductive toxicity and thus influence the health of individuals including the next generation. Genotoxicity testing is therefore an important part of the safety assessment of new compounds and products including nanomaterials (Doak et al., 2012; Dusinska et al., 2011). It is clear that some nanomaterials are genotoxic; but which properties are responsible for their toxicity and what is the mechanism of

Correspondence: Dr. Maria Dusinska, Health Effects Laboratory, NILU – Norwegian Institute for Air Research, Instituttveien 18, 2027 Kjeller, Norway. Tel: +4763898157. E-mail: maria.dusinska@nilu.no

genotoxicity are still not well understood. Published data show that there are direct and indirect mechanisms of NP-induced genotoxicity (Bhabra et al., 2009; Donaldson et al., 2010; Magdolenova et al., 2014; Sood et al., 2011). NPs taken up by cells can come into direct contact with the genetic material causing physical or chemical damage. However, the most likely mechanism of NP-induced genotoxicity is indirect, *via* intermediate biomolecules that either are involved in normal genome function or cell division or attack DNA, causing DNA injury or chromosomal malformations. Oxidative stress is considered to be the key indirect mechanism of NP-induced genotoxicity. Induction of reactive oxygen species (ROS) by NPs can cause DNA injury as well as damage to lipids, proteins and other cellular components. The secondary mechanisms of NP-induced genotoxicity *in vivo* are also mediated by extracellular ROS *via* the inflammatory responses of macrophages and neutrophils (Magdolenova et al., 2014; Stone et al., 2009).

The comet assay (single cell gel electrophoresis) is the most commonly used method in nanogenotoxicity studies (Magdolenova et al., 2014). Several tests successfully used for hazard assessment of ordinary chemicals seem to be unsuitable or need adaptation for application with nanomaterials. For example, the Ames test is not well suited for NP-induced genotoxicity testing, due to the size of the bacteria and the limited or nil uptake of NPs through the cell wall (Dusinska et al., 2012). Primary and secondary properties of NPs can result in interference with standard toxicity tests, and this has been demonstrated for a range of *in vitro* cell viability and cytotoxicity assays and for oxidative stress detection (Aranda et al., 2013; Guadagnini et al., 2015a; Kroll et al., 2012). In the case of genotoxicity, interference was reported with the micronucleus test as cytochalasin B (used in this assay) inhibits endocytosis and may prevent uptake of NPs (Magdolenova et al., 2012a). There have been concerns about the comet assay either for possible false-positive results due to presence of NPs in the gel (Rajapakse et al., 2013; Stone et al., 2009) or for possible interaction of NPs with lesion-specific enzymes resulting in false-negative results (Kain et al., 2012). However, the study of Magdolenova et al. (2012a) showed that incubation of nuclei with NPs does not lead to any decrease in the yield of formamidopyrimidine (FPG)-sensitive sites. No interference with the repair enzyme FPG was found with five NanoTEST NPs tested when present in the gel (Magdolenova et al., 2012a). The comet assay is able to distinguish between genotoxic and non-genotoxic compounds and is sufficiently sensitive to detect DNA damage induced by NPs (Doak et al., 2012; Landsiedel et al., 2009; Magdolenova et al., 2014). The alkaline version of the comet assay detects strand breaks (SBs) and alkali-labile sites. Several modifications of the method are available that can detect different genotoxic lesions such as cross-links or, by using lesion-specific enzymes, alkylated or oxidized DNA bases (Collins, 2004; Dusinska & Collins, 1996, 2008). These modifications increase versatility, sensitivity and specificity of the assay and enable us to study mechanisms of how NPs induce genotoxicity. Photogenotoxic effects of NP can also be measured in combination with ultraviolet radiation (Dodd & Jha, 2009; Reeves et al., 2008; Vevers & Jha, 2008). Furthermore, the assay has the advantage that it can be carried out both *in vitro* and *in vivo*, in proliferating as well as non-proliferating cells. Any nucleated cells, from different tissues or organs, can be evaluated. The comet assay can thus assess both cell- and tissue-specific DNA damage induced by NPs. In this study, we investigated *in vitro* genotoxicity of six NanoTEST NPs on primary cells and cell lines originating from blood, lung, liver, kidney, brain and placenta.

Materials and methods

NPs

Polylactic glycolic acid (PLGA) polyethylene oxide (PEO) copolymer was provided by Advanced In Vitro Cell Technologies (Barcelona, Spain) as a 10 mg/mL suspension in water.

Titanium dioxide (TiO_2), an anatase/rutile powder of 21 nm nominal size, material type NM-105 received from the European Commission – Joint Research Centre (Ispra, Italy), was manufactured by Evonik (Essen, Germany) and marketed as Aeroxide TiO_2 P-25. TiO_2 NPs were provided as nanopowder, and the particles were dispersed in culture medium by the following two methods (for details see Magdolenova et al., 2012b): (a) TiO_2 UPdisp: stock solutions of TiO_2 NPs were prepared by suspending NPs in culture media without foetal bovine serum (FBS). The suspension was sonicated using an induced ultrasonic probe at 60 W for 3 min or (b) TiO_2 Vedisp: to prepare 1 mL of stock solution, 1 mL of 20% FBS in phosphate-buffered saline (PBS) was added to 5 mg of TiO_2 NPs in a microcentrifuge tube. The dispersion was sonicated with an ultrasonic probe for 15 min at 100 W.

Nano-magnetite water dispersions (uncoated (U-Fe_3O_4) or coated with oleic acid (OC-Fe_3O_4)) were purchased from Plasmachem (Berlin, Germany). NPs were provided as a 28 mg/mL (U-Fe_3O_4) or 260 mg/mL (OC-Fe_3O_4) suspension in water and diluted in PBS to obtain a final stock concentration of 2 mg/mL.

Amorphous silica was purchased from Corpuscular INC (Cold Spring Harbor, NY) as a water dispersion of rhodamine-labelled 25 nm (Fl-25 SiO_2) or 50 nm (Fl-50 SiO_2) diameter particles.

After vortexing, all NPs were diluted in culture medium to working concentrations corresponding to 75 μg/cm^2.

Characterisation of NPs

NPs were fully characterised as previously described (Guadagnini et al., 2015a; Kazimirova et al., 2012; Magdolenova et al., 2012b, 2015) by a combination of analytical techniques. Briefly, the mean average size of primary particles was determined by Transmission Electron Microscopy with a Jeol 3010 transmission electron microscope (JEOL Ltd., Tokyo, Japan) operating at 300 kV. Surface area and pore volume were obtained by nitrogen adsorption on a Micromeritics ASAP2000 Accelerated Surface Area and Porosimetry System at an adsorption temperature of −196 °C, after pretreating the sample under high vacuum at 300 °C for 2 h (Brunauer et al., 1938). Actual concentrations of Fe_3O_4 NPs in batches provided were determined by atomic absorbance spectroscopy after acid digestion of sample aliquots. Dynamic light scattering (DLS) analysis and zeta potential of provided dispersions, as well as of NP dispersions in culture medium, were determined with a Submicron Particle Sizer Nicomp 380 equipped with a 35 mW He-Ne laser 632.8 nm laser diode and photodiode detector set at 90° (PSS, Port Richey, FL). Suspensions of NPs investigated in biological media were freshly prepared according to separate protocols specifically developed for each NP type. Stock dispersion was added to cell culture medium to achieve a 75 μg/cm^2 working solution and concurrently analyzed by DLS.

Cell lines and cell cultures

Primary cells: human lymphocytes, rat hepatocytes and Kupffer cells and stable cell lines: human lymphoblastoid TK6 cells, human kidney HEK293, monkey kidney COS-1 cells, human brain endothelial cells human cerebral endothelial cells (HCECs),

bronchial 16HBE14o cells and human BeWo b30 placental cells were used, as detailed below. All cells were maintained at 37 °C and pH 7.4 with 5% CO_2 in a humidified atmosphere.

Hepatocytes were isolated from rat livers using a two-step tissue microperfusion technique (Berry & Friend, 1969). Livers were cannulated through the accessible vessels with a 1-mm diameter needle. A balanced salt solution was injected to clean the organ using a peristaltic pump. After washing, a solution containing collagenase was added for liver disintegration. The cell suspension obtained was filtered and centrifuged and, after two washings to remove collagenase, pelleted cells were resuspended in Ham's F-12/Leibovitz L-15 (1:1) culture medium supplemented with 2 mM glutamine, 170 µg/mL sodium selenite, 2% newborn calf serum, 50 mU/mL penicillin, 50 µg/mL streptomycin, 0.2% bovine serum albumin and 10 nM insulin (Gómez-Lechón & Castell, 1998) and cultured on fibronectin-coated plates.

Kupffer cells were obtained by centrifuging the (initial) supernatant obtained after hepatocyte isolation. After this, cell pellets were suspended in cold Krebs-Ringer and loaded onto a Percoll gradient (50%/25%). After centrifugation, the cells were distributed in four distinct zones in the gradient. The intermediate zone enriched in Kupffer cells and sinusoidal endothelial cells was harvested. After several washes to remove Percoll, cells were resuspended in cultured medium (RPMI supplemented with 10% FCS and 2 mM glutamine) and then plated on plastic dishes. After 1-h incubation, only Kupffer cells attached well to the plates (Titos et al., 1999).

Human peripheral lymphocytes were isolated from blood of 13 volunteers (six women and seven men; age 40–50 years). Volunteers participating in the study were not exposed to any known pollutants or mutagens. Blood was collected by venepuncture from fasted subjects and aliquoted in EDTA tubes. All study participants signed an informed consent form. This study was approved by the Ethical Committee of the Slovak Medical University in Bratislava. Peripheral blood mononuclear cells (mostly lymphocytes) were isolated by gradient centrifugation on Lymphoprep. The cell suspension (2×10^6 cells per mL) in RPMI medium was dispensed into a 96-well plate (100 µL per well; growth area of 0.32 cm^2); 25 µL of tested NP dispersion was added per well with cell suspension. Cells were treated with 0.12–75 µg/cm^2 NPs for 4 h (in RPMI medium with 10% FCS) and for 24 h (in RPMI medium with 20% FCS).

TK6 human lymphoblastoid cells (obtained from the European Collection of Cell Cultures – ECACC, Cat. no. 95111735) were maintained in RPMI 1640 (Cat. no. R8758, Sigma Aldrich, Saint-Quentin Fallavier, France) culture medium supplemented with 10% (v/v) heat-inactivated FBS, 100 U/mL penicillin and 100 µg/mL streptomycin. Cells were grown in suspension routinely diluted to $2 \times 10^5 - 1 \times 10^6$ cells/mL. For the experiment, TK6 cells were sub cultured at a density of 4×10^5 cells/mL into six-well plates with 2 mL of culture medium containing freshly dispersed NPs.

HCECs were a gift from Prof. D. Stanimirovic (Ottawa, Ontario, Canada) and were grown in DMEM medium (Invitrogen, Cergy-Pontoise, France), containing 4.5 g/l glucose, 10% FBS and penicillin/streptomycin (Halamoda Kenzaoui et al., 2012a,b).

16HBE14o, differentiated SV-40 transformed human bronchial epithelial cell line was kindly provided by Dr. Gruenert (Colchester, Vermont University) and cultured as previously described (Guadagnini et al., 2015a). Cells were grown in DMEM/F12 (Invitrogen) supplemented with 1% GlutaMAX (Invitrogen) and 2 % Ultroser® G (UG, Biosepra, Garenne, France). Cells were cultivated on plastic supports (Corning Costar, Brumath, France) coated with human fibronectin (0.01 mg/mL, Sigma Aldrich, Saint-Quentin Fallavier, France), bovine serum albumin (0.1 mg/mL, Sigma Aldrich, Saint-Quentin Fallavier, France) in basic LCH medium (Invitrogen).

The kidney fibroblast cell lines Cos-1 and *HEK 293* were maintained in DMEM medium (1000 mg/L glucose) (Sigma Aldrich, Saint-Quentin Fallavier, France) containing 10% FBS, 100 U/mL penicillin and 100 mg/mL streptomycin.

The placental choriocarcinoma cell line, BeWo b30, was obtained from Prof. Harry McArdle (Rowett Research Institute, Aberdeen, Scotland) with permission from Dr. Alan Schwartz (Washington University, St. Louis, MO). Cells were cultured in complete medium supplemented with 10% FBS as described previously (Cartwright et al., 2012). The BeWo b30 cell lineage has been authenticated by the European Cell and Culture Collection (Jones et al., 2006).

All cells were cultivated in 25 cm^2 or 75 cm^2 flasks at 37 °C and 5% CO_2 in a humidified atmosphere, and culture medium was changed every 2–5 days. Adherent cells were routinely sub-cultured every 2–7 days by detaching cells with 0.05%-trypsin EDTA (ethylenediaminetetracetic acid) (2 mg/mL, Invitrogen) or with 0.4% trypsin–EDTA (Sigma-Aldrich, Gillingham, UK) in the case of BeWo cells. Enzymatic activity of trypsin was inhibited by the addition of 10% FCS/FBS. Cells grown in suspension were routinely diluted to $2 \times 10^5 - 1 \times 10^6$ cells/mL.

Cells were exposed to NPs at five concentrations: 0.12, 0.6, 3, 15 and 75 µg/cm^2, which correspond to 0.57, 2.9, 14.4, 72.0 and 360.2 µg/mL (calculated for six-well plates with 2 mL medium per well; the actual concentration per mL differs depending on size of the culture plate or well) for a short period of 30 min, for 1, 2, 4 h and for 24 h. After treatment, the cytotoxicity tests and the comet assay were performed.

The alkaline comet assay for detection of DNA SBs and oxidised purines

After exposure, cells were washed, re-suspended and embedded in 120 µL of 1% low melting point agarose in PBS at 37 °C. Two gels for each sample were placed on a glass microscope slide (precoated with melted 0.5% normal melting point agarose in H_2O) and covered with 15 × 15 mm coverslips, placed for 5 min at 4 °C to solidify; coverslips were removed and slides were immersed in lysis solution (2.5 M NaCl, 0.1 M EDTA, 10 mM Tris-HCl, pH 10, with 1% Triton X-100 added immediately before use) for 1 h–24 h at 4 °C. During lysis, cell membranes, cytoplasm and most chromatin proteins are removed, leaving free nuclear DNA in the agarose gel as nucleoids. For FPG enzyme treatment, slides were washed three times for 5 min in cold (4 °C) FPG reaction buffer (40 mM HEPES, 0.1 M KCl, 0.5 mM EDTA, 0.2 mg/mL bovine serum albumin, pH adjusted to 8.0 with KOH); 30 µL of FPG solution (final dilution − 1/3000) or reaction buffer alone (as a negative control) was added to the gel and covered with a cut square of Parafilm. Slides were incubated in a moist box for 30 min at 37 °C, placed at 4 °C for 5 min and the Parafilm removed. FPG was provided by Professor Andrew Collins (Department of Nutrition, University of Oslo, Norway). After lysis or enzyme incubation, the slides were placed on a platform in a horizontal electrophoresis tank, immersed in cold (4 °C) electrophoresis solution (0.3 M NaOH, 1 mM EDTA) for 20 min and subjected to electrophoresis at 25 V (0.8 V/cm, approximately 300 mA) for 20 min. After neutralization for 10 min in PBS, followed by 10 min in water, slides were dried at room temperature or dehydrated with alcohol.

As positive controls, cells were treated with 50 µM H_2O_2 in PBS (4 °C) for 5 min on ice (for SBs) and with 1 µM photosensitiser Ro 19-8022 (kindly provided by Hoffmann-LaRoche) in PBS together with visible light (for 8-oxoguanine and other oxidised purines). For Kupffer cells and hepatocytes,

cells exposed to 7000 μW/cm^2 UV radiation for 2 min were employed as positive controls.

As negative controls, untreated cells cultivated in the same medium as NPs were used. In addition, Endorem® dextran-coated iron oxide NPs (Guerbet, Villepinte, France; 11.2 mg/mL) dispersed in water were used as nanomaterial-based negative control (data not shown). The particles are composed of several iron oxide cores (diameter 4–6 nm) embedded in a dextran coating with an estimated hydrodynamic size of 80–150 nm and a neutral surface charge (Cengelli et al., 2006).

Slides were stained with SYBR Gold (Invitrogen), ethidium bromide or DAPI, covered with a cover slip and viewed by fluorescence microscopy with a CCD camera. Quantification was carried out using the image analysis system Comet Assay IV (Perceptive Instruments Ltd., Suffolk, UK), scoring 50 comets per gel and minimum 100 comets per sample and recording the % DNA in tail. Comets from BeWo b30 and HCEC cells were analyzed visually; each comet was given a score of between 0 (no tail) and 4 (small head, almost all DNA in tail), and the overall score for 100 comets was obtained by summing the individual scores. The visual score, expressed in arbitrary units, therefore ranged from 0 to 400. All data expressed in arbitrary units have been transformed to % DNA in tail using the equation (Collins et al., 1997):

$$VS = (4.96 \times \%T) + 4.42 \text{ and so}$$

$$\%T = \frac{(VS - 4.42)}{4.96}$$

Subtracting the score for ''SBs and alkali-labile sites'' from the ''FPG incubation'' score gives a measure of FPG-sensitive sites in the DNA, i.e. oxidised purines, mainly 8-oxoguanine.

Cytotoxicity

Relative growth activity and trypan blue exclusion (TBE) was measured in TK6 and Cos-1 (Magdolenova et al., 2015). In lymphocytes, cytotoxicity was measured by TBE; cytotoxicity in Kupffer cells, hepatocytes and HCEC was measured using the MTT assay (Halamoda Kenzaoui et al., 2012a), in 16HBE14o by the WST-1 assay and in BeWo b30 cells by the WST-1 assay and TBE (Correia Carreira et al., 2015; Guadagnini et al., 2015b).

Statistical analysis

The data were described using tables and graphs as appropriate and summary statistics of means and variability were calculated, i.e. mean values with standard deviation (means ± SD) or standard error of mean (means ± SEM).

Statistical regression modelling was carried out using the GenStat statistical software package (VSN International, 2011). Two kinds of statistical modelling analyses were performed. The first set of analyses looked at differences between nanomaterials by examining the dose-response association between concentration and SBs or concentration and net FPG-sensitive sites and determining whether it varied among nanomaterials. The second set of analyses examined the relative toxicity of NPs on the different cell lines used. As not all cell lines were used with all nanomaterials, we looked at the differences in cell lines separately for each nanomaterial (i.e. we modelled cell line within nanomaterial). All analyses were performed with and without adjustment for time period of the genotoxicity tests; the inclusion or exclusion of this variable did not affect the dose-response associations. Models were fitted on a log-scale of the response, which provided a better fit to the data.

Statistical analysis for BeWo b30 cells was additionally performed using SPSS v18 for Windows (IBM UK Ltd., Portsmouth, UK), and results for the comet assay analysed by one-way analysis of variance (ANOVA) followed by Games Howell multiple comparison procedure. A statistical probability of $p < 0.05$ was considered significant (Bright et al., 2011).

Results

Six laboratories from the NanoTEST consortium performed the comet assay following the same experimental design and procedures. PLGA-PEO, TiO_2, U-Fe_3O_4, OC-Fe_3O_4, Fl-25 SiO_2 and Fl-50 SiO_2 NPs from single batches were tested for genotoxicity. An overview of all comet assay analyses is listed in Table 1. Primary cells and stable cell lines representing six different organs or tissues were treated with NPs for a short period of 30 min, 1, 2 or 4 h (data not shown for lymphocytes and stable cell lines) and for 24 h in a concentration range from 0.12 to 75 μg/cm^2. Primary cultures of rat Kupffer cells were exposed only for a shorter period of time (up to 4 h) due to cell toxicity when exposed to NPs for longer periods of incubation. Except for the lung model where only three NPs (TiO_2, OC-Fe_3O_4 and Fl-25 SiO_2) were evaluated, and HCEC where five from six NPs (all but Fl-50 SiO_2) were analysed, for all representative cell models, all six NPs were assessed for genotoxicity using the comet assay (Table 1). For a kidney model, monkey COS-1 cells were selected as an appropriate cell type. In addition, TiO_2NPs (in both TiO_2 Vedisp and TiO_2 UPdisp dispersions) were analysed also with human kidney HEK293 cells. IC_{50} values were calculated for each NP and for each cell line when possible. Table 2 summarises the results of the analysis of the effect of concentration on SBs and of how it differs between cell lines with NPs. The results of the analysis show that SBs were significantly associated with time, concentration and NPs. It also shows that the association between concentration and SBs varies significantly between NPs, but the dose-response does not vary significantly between cell lines within NPs ($p > 0.05$). Results also show that the strongest positive dose-response associations occurred for OC-Fe_3O_4, and for TiUPdisp (Figure 1). However, OC-Fe_3O_4 NPs induced high toxicity and were the most toxic of all the studied NPs. The IC_{50} values measured at 24-h exposure for OC-Fe_3O_4 NPs were for 16HBE14o cells 6.76, for BeWo b30 cells 15.31, for TK6 35.53 and for HCEC cells 54 ug/cm^2. Positive dose-response associations were also seen for U-Fe_3O_4, Fl-25 SiO_2 and TiO_2Vedisp. No dose-response association was seen for PLGA-PEO or Fl-50 SiO_2. For FPG-sensitive sites, only the TiO_2 NPs show weak evidence of a positive dose-response association. Similar to the SBs there was no significant difference in dose-response associations among cell lines.

We further analysed differences between cell lines for the two NPs generating the strongest genotoxic response – OC-Fe_3O_4 and TiO_2UPdisp (Table 3). The results summarised in the ANOVA table show that when considering only OC-Fe_3O_4, the dose-response association varies significantly among cell lines. The strong positive dose-response associations occur for TK6, Cos-1, human lymphocytes and BeWo b30 as can be seen in Figure 2(a). No dose-response association was seen for HCEC, Kupffer cells or rat hepatocytes. Results of the analyses for TiO_2UPdisp show that the dose-response did not vary significantly among cell lines ($p > 0.05$), despite the difference in the slope of the curve (Figure 2b).

Exposure of TK6 cells to U-Fe_3O_4, PLGA-PEO, Fl-25 SiO_2 and Fl-50 SiO_2 and Endorem NPs did not cause significant genotoxicity. TiO_2 NPs induced DNA damage in TK6 cells (Figure 2b) dependent on the dispersion protocol used (Magdolenova et al., 2012b). OC-Fe_3O_4 NPs increased SBs and oxidized bases (Magdolenova et al., 2015). Comet assay data on peripheral blood lymphocytes confirmed the results on TK6 cells.

Table 1. Overview of comet assay results (strand breaks); assay performed in cells and cell lines of human (h), monkey (m) or rat (r) origin derived from six different organs, after exposure to NanoTEST nanoparticles.

Organ	Cells	TiO_2 V/UPdisp	OC-Fe_3O_4	U-Fe_3O_4	PLGA-PEO	Fl-25 SiO_2	Fl-50 SiO_2
Lung (h)	16HBE14o	–/Y	Y	–	–	Y	–
Brain (h)	HCEC	–/Y[1]	Y[1]	Y[1]	Y	Y	–
Blood (h)	TK6	Y/Y[2]	Y[3]	Y[3]	Y[4]	Y	Y
	Lymphocytes	Y/–	Y[3]	Y[3]	Y[5]	Y	Y
Placenta (h)	BeWo b30	Y/–	Y	Y	Y	Y	Y
Kidney (m/h)	Cos-1	Y/Y[2]	Y[6]	Y[6]	Y	Y	Y
	HEK293	Y/Y	–	–	–	–	–
Liver (r)	Kupffer	–/–	Y	Y	Y	Y	Y
	Hepatocytes	–/Y	Y	Y	Y	Y	Y

All laboratories followed the same experimental design, handling procedures, exposure and experimental conditions. All cells and cell lines were exposed to nanoparticles (NPs) from the same batch using the same dispersion protocols with five concentrations: 0.12, 0.6, 3, 15 and 75 μg/cm^2. TiO_2 NPs were dispersed following Vdisp (Venice) or Updisp (University of Paris) protocols. Except for liver cells where NPs were applied only for shorter periods (30 min to 4 h), all other cells were exposed for both a short period of 30 min to 1, 2, 4 h and in all cases for 24 h as standard.

References to published genotoxicity results (short and/or long term exposures): Halamoda-Kenzaoui et al., 2012a; Magdolenova et al., 2012b; Magdolenova et al., 2015; Kazimirova et al., 2012; Tulinska et al., 2015; and Harris et al., 2015.

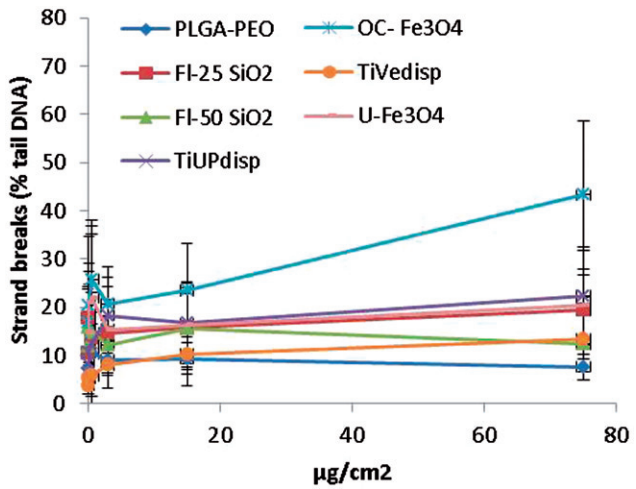

Figure 1. Comparison of genotoxic effect of metalic (OC-Fe_3O_4, U-Fe_3O_4, TiO_2 and Fl-25 SiO_2 Fl-50 SiO_2) and polymeric nanoparticles (PLGA-PEO NPs) after 24-h exposure *in vitro*. Strand breaks are expressed as % DNA in tail (mean ± SEM) versus concentration of nanomaterial. The slope of the dose-response line separately for each nanomaterial is shown also on table below. The strongest positive dose-response associations occur for OC-Fe_3O_4, and for TiUPdisp. Significant positive dose-response associations were also seen for U-Fe_3O_4, Fl-25 SiO_2 and TIVedisp with less steep slopes with concentration. No dose-response association was seen for PLGA-PEO or Fl-50 SiO_2.

Nanomaterial	Coefficient (per μg/cm^2)	s.e.	Coeff/s.e. (*t*-value)
OC-Fe_3O_4	0.0083	0.0012	6.44
U-Fe_3O_4	0.0020	0.0011	1.94
PLGA-PEO	–0.0013	0.0011	−1.23
Fl-25 SiO_2	0.0021	0.0011	1.97
Fl-50 SiO_2	0.0016	0.0011	1.45
TIVedisp	0.0029	0.0012	2.53
TiUPdisp	0.0053	0.0019	2.85

HCEC (endothelial) cells gave a similar response to cells of blood origin (TK6) or from kidney showing TiO_2 and OC Fe_3O_4 NP-induced DNA damage (Figure 2a,b).

DNA oxidation studied in TK6 cells, lymphocytes and kidney cells showed that OC-Fe_3O_4 NPs induced a mild but significant increase in oxidative DNA lesions (Magdolenova et al., 2015) additional to SBs (Figure 2a). Fl-25 SiO_2 (Figure 2c) and Fl-50

Table 2. Accumulated analysis of variance for strand breaks (SBs) (a) and net FPG-sensitive sites (b). Effects of time, concentration and cell line within nanomaterial.

Variable	Degrees of freedom	Sum of squares	Mean square	Variance ratio	*F* probability
(a) SBs					
Time	1	27.39	27.39	239.44	<0.001
Concentration	1	6.40	6.40	56.0	<0.001
Nanomaterial	6	7.26	1.21	10.6	<0.001
Cell line within NM	40	164.02	4.10	35.8	<0.001
Dose: Nanomaterial concentration	6	9.82	1.64	14.3	<0.001
Cell line within NM	40	4.34	0.11	1.0	0.564
Residual	1347	154.08	0.11		
Total	1441	373.32	0.26		
(b) Net FPG-sensitive sites					
Time	1	55.49	55.49	52.2	<0.001
Concentration	1	0.94	0.94	0.9	0.347
Nanomaterial	6	17.66	2.94	2.8	0.001
Concentration: Nanomaterial	6	16.18	2.70	2.5	0.19
Residual	950	1010.33	1.06		
Total	964	1100.61	1.14		

SiO_2 NPs did not cause a significant increase in SBs or in oxidized bases (data not shown). TiO_2 NPs were assessed also in HEK 293 cells with the same impact; induction of DNA damage depended on dispersion (Figure 2b; Magdolenova et al., 2012b). TiO_2 NPs were the only NPs dose-dependently inducing genotoxicity in hepatocytes (Figure 2b). In contrast, all tested NPs but PLGA-PEO NPs caused DNA damage in Kupffer cells even after a short exposure time. Data correlated well with those for NP toxicity in these cells. Thus, Kupffer primary cultures were found to be more susceptible to NP toxicity than hepatocytes, pointing to different internalization patterns of the NPs in the two cell types. Kupffer cells internalized SiO_2 NPs after a 4-h period while uptake in hepatocytes only occurred after 24-h exposure (data not shown). Both cell types had higher background levels of DNA damage compared to other cells (Figure 2). Similarly, 16HBE cells appeared to be very sensitive cells with a higher background level of DNA damage and were thus unable to show a significant positive genotoxicity response for OC Fe_3O_4 and TiO_2 NPs (Figure 2a,b). From three NPs analysed in the human lung cells, the DNA damage was significantly induced only after Fl-25 SiO_2 NP exposure (Figure 2c).

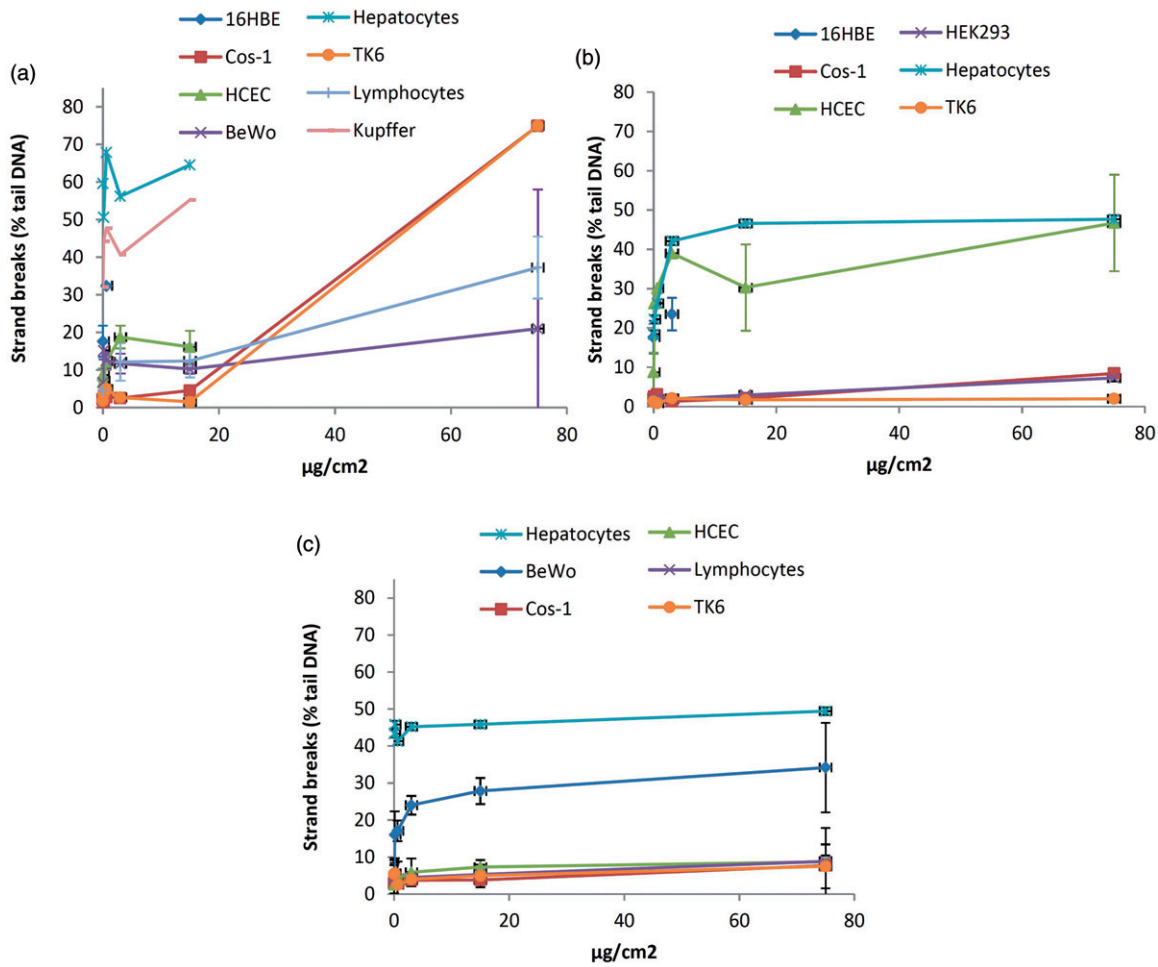

Figure 2. DNA damage measured by the comet assay in various cells and cell lines (16HBE, BeWo b30 Cos-1, HCEC, TK6 cells, human lymphocytes, rat hepatocytes and Kupffer cells) after 24-h exposure to (a) OC-Fe_3O_4; (b) TiO_2UPdisp and (c) Fl-25 SiO_2 nanoparticles. DNA damage is expressed as strand breaks. Data are presented as % DNA in tail of mean values from replicate experiments (mean ± SEM).

Figure 3 shows an example of the response to different NPs for a selected cell line, BeWo b30. Exposure to U-Fe_3O_4, Fl-25 SiO_2 and TiO_2Vedisp did not cause a significant alteration in cell viability as shown by TBE, but exposure to both OC- and U-Fe_3O_4 NPs at 3–75 µg/cm^2 did result in significant levels of cytotoxicity as determined by the WST-1 assay. Whilst there appears to be an increase in DNA breaks with increased Fl-25 SiO_2 concentration, the results are not significant, as is also the case for U-Fe_3O_4 exposure. In contrast, exposure to TiO_2Vedisp NPs at 0.6–75 µg/cm^2 generated a significant level of SBs in the absence of significant cell membrane damage or cytotoxicity. Whilst significant increase in SBs was observed following exposure to 0.6 and 75 µg/cm^2 of OC-Fe_3O_4 NPs, the outcome is confounded by evidence of significant cell damage at the highest concentration, which highlights the importance of determining genotoxicity at non-cytotoxic concentrations.

In summary, two endpoints, SBs and net FPG-sensitive sites, were analysed. For both endpoints, there were differences in dose-response among different NPs. However, when all NPs were included in the analysis, no differences were seen among cell lines within NPs. For SBs, a strong positive association was seen between concentration and response for OC-Fe_3O_4 and TiO_2UPdisp. For net FPG-sensitive sites, a mild positive association was only found for TiO_2 NPs. All types of cells were able to demonstrate a positive genotoxic response to NPs. The most sensitive cells appear to be primary rat liver cells. The TK6 cells, human lymphocytes, Cos-1 and BeWo b30 cells seem the most reliable for detecting a dose-response to NPs.

Discussion

Genotoxic compounds can be mutagenic and thus potentially carcinogenic. It is therefore of particular interest to develop suitable genotoxicity assay for NPs as an essential part of a testing strategy. The genotoxicity endpoint was one of the cross-cutting topics in NanoTEST, together with oxidative stress, cytotoxicity and inflammation/immunotoxicity. Within NanoTEST, the following assays and endpoints were used to evaluate genotoxicity of NPs: the micronucleus assay to detect mutagenicity, clastogenicity and aneugenicity (Kazimirova et al., 2012; Tulinska et al., 2015); the comet assay to detect SBs (Halamoda-Kenzaoui et al., 2012a; Harris et al., 2015; Kazimirova et al., 2012; Magdolenova et al., 2012b, 2015; Tulinska et al., 2015), the FPG-modified comet for oxidised DNA lesions (8-oxoGuanine and Fapy derivates) (Harris et al., 2015; Kazimirova et al., 2012; Magdolenova et al., 2012a,b, 2015); phosphorylation of Histone2A.X (pH2AX) for double SBs (Harris et al., 2015).

The comet assay is a simple and reliable test especially useful for nanomaterial testing due to its robustness and it is the most used assay in nanogenotoxicology studies (Magdolenova et al., 2014). Medium and high throughput versions of the assay have already been applied in nanomaterial testing, allowing analysis of large numbers of NPs in a relatively short time

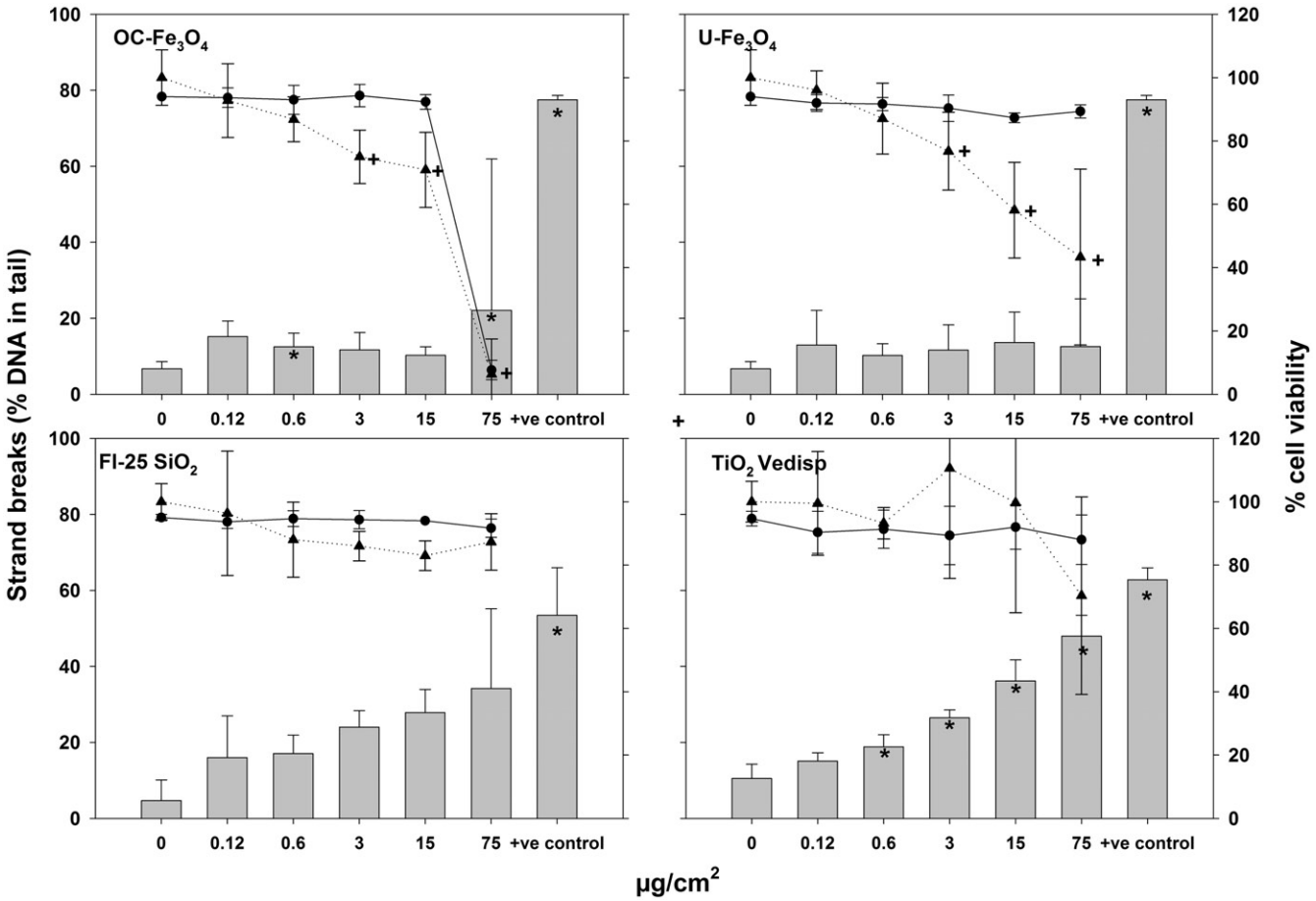

Figure 3. Genotoxicity, cell membrane damage and cytotoxicity after exposure of BeWo b30 cells to OC-Fe_3O_4, U- Fe_3O_4, Fl-25 SiO_2 or TiO_2 Vedisp NPs for 24 hours. For each treatment, a total of 4×10^4 exposed cells were embedded in 120 µl of 1% low melting point (LMP) agarose with two replicate gels set on each slide. Data are shown as mean and standard deviation of three independent experiments. Statistical analysis was performed using SPSS v18 for Windows, and results for the comet assay analysed by one-way analysis of variance (ANOVA) followed by Games Howell multiple comparison procedure. A statistical probability of $p < 0.05$ was considered significant (*for comet bar, + for WST-1 cytotoxicity). Strand breaks are shown by filled grey vertical bars with the arbitrary scale on the left axis. Cell viability was determined by trypan blue exclusion (TBE) under the same exposure conditions as for the comet assay. TBE is shown by the line graph with filled circles with the % cell viability scale shown on the right-hand axis. Cytotoxicity was determined by the WST-1 assay as shown by the dotted line graph with filled triangles using the same % cell viability scale on the right-hand axis.

Table 3. Accumulated analysis of variance for strand breaks (SBs) induced by: (a) OC-Fe_3O_4; (b) TiO_2UPdisp. Effects of time, concentration and cell line.

Variable	Degrees of freedom	Sum of squares	Mean square	Variance ratio	*F* probability
(a) OC-Fe_3O_4					
Time	1	2.21	2.21	26.9	<0.001
Dose	1	9.07	9.07	110.3	<0.001
Cell line	7	21.76	3.11	37.8	<0.001
Dose Cell line	7	1.41	0.20	2.4	0.020
Residual	213	17.51	0.08		
Total	229	51.95	0.23		
(b) TiO_2UPdisp					
Time	1	6.05	6.05	108.0	<0.001
Dose	1	1.74	1.74	31.0	<0.001
Cell line	5	15.75	3.15	56.2	<0.001
Dose. Cell line	5	0.44	0.09	1.6	0.181
Residual	75	4.20	0.06		
Total	87	28.18	0.32		

(Harris et al., 2015). The assay is sensitive enough to detect DNA damage induced by NPs. A possibility of artefactual damage occurring if NPs remain in the gel interacting directly with non-nucleosomal DNA and potentially causing additional DNA damage was raised by Stone et al. (2009), Karlsson (2010) as well as Rajapakse et al. (2013). We tested NanoTEST NPs used in this study for possible interference and found none for all tested NPs (Magdolenova et al., 2012a). When TiO_2, OC-Fe_3O_4NPs, U-Fe_3O_4, Fl-25 SiO_2 and Fl-50 SiO_2 NP were present at high concentration in the gel with the lysed cells, we found no increase in SBs. A modification of the comet assay, involving digestion of DNA with lesion-specific endonucleases – such as FPG, which detects oxidised purines – raised a different concern about artefacts resulting from the presence of residual NPs with the DNA of lysed cells. In this case, there is a risk of false-negative results if NPs interfere with the FPG reaction. An interaction of NPs with FPG has been reported by Kain et al. (2012) for two tested NPs; they suggested that this interaction could lead to underestimation of DNA oxidation caused by NPs. These authors mixed NPs directly with FPG at high concentrations, which does not correspond to experimental conditions in the comet assay; even so, FPG was efficient after mixing with three of the five NPs. Our study (Magdolenova et al., 2012a) showed that incubation of nuclei (treated with a known 8-oxoguanine-inducing agent) with NPs does not lead to any decrease in the yield of FPG-sensitive sites. No interference with FPG was found with all five NPs tested when present in the gel. In spite of our finding, we suggest that thorough rinsing and testing for possible interference

of NPs in the gel, using both untreated cells and cells exposed to a known genotoxic compound (causing DNA SBs as well as oxidized DNA lesions), would seem to be a sensible precaution to be sure that no overestimation or underestimation of damage is occurring.

Concentrations used for genotoxicity studies should be realistic, i.e. relevant to possible exposures. For the comet assay, recommended concentrations should correspond to at least 60%–80% viability, since DNA breaks can be secondary effects of cytotoxicity and so could give false positives (Dusinska et al., 2013). Thus, together with the comet assay, cytotoxicity should always be tested with the same cells (and ideally in the same experiment). The most reliable assays are relative growth activity and colony-forming ability addressing cell death as endpoint. MTT, WST-1 and other colorimetric assays are prone to interference by the NPs but are reliable as long as the interference is tested (Guadagnini et al., 2015a).

Interestingly, statistical analysis showed that all the cell lines and primary cells studied are able to identify genotoxic responses since when analysing all NPs, no significant difference was found between cells. However, primary cells isolated from rat liver as well as 16HBE cells appeared to be more sensitive than other cell types. These cells also showed higher background levels of DNA damage and therefore are less suitable as standard models for testing NP genotoxicity until conditions for this application are further standardised. When comparing cell lines of different origin, results show that endothelial cells derived from central nervous system (HCEC) gave a similar response to cells of blood origin (TK6) or cells from kidney in terms of TiO_2 and OC Fe_3O_4 NP-induced DNA damage. Similarly, in BeWo b30 cells, the comet assay results showed a significant positive response to TiO_2 Vedisp and OC-Fe_3O_4 NPs though only at one non-cytotoxic concentration (Figure 3). PLGA-PEO did not cause any DNA damage in any of the cell lines or primary cells and thus a negative genotoxic effect was demonstrated in all cell types. The TK6 cells, human lymphocytes, BeWo b30 and kidney cells seem to be the most reliable for detecting a dose-response.

Our results demonstrate that the *in vitro* induction of DNA damage is associated with NP type, time of exposure and concentrations. OC-Fe_3O_4 and TiO_2 UPdisp appear to generate the strongest response, with both showing a positive dose-response association in contrast to PLGA-PEO or Fl-50 SiO_2 NPs where there was no positive effect. The positive genotoxic effect of OC-Fe_3O_4 was not related to oleic acid as shown by Magdolenova et al. (2015). While OC-Fe_3O_4 and TiO_2 UPdisp would make good positive controls for evaluation of nanomaterial genotoxicity, the response to OC-Fe_3O_4 was cell line-dependent and exposure caused significant cytotoxicity in contrast to TiO_2 UPdisp. PLGA-PEO appears to be suitable as a negative control across all cell types. Generally, for screening of NPs for genotoxicity, any standard cell lines such as were used in our study are suitable for detecting DNA damage. To simulate organ specificity, cell lines originating from a particular organ can be used. Human primary lymphocytes can also be used as a surrogate target organ option.

Acknowledgements

We wish to acknowledge the additional technical support from Norma Houssein to generate the TiO_2 NP comet assay data.

Declaration of interest

The authors declare that there is no conflict of interest.

The work was supported by EC FP7 NanoTEST [Health-2007-1.3-4], contract no: 201335, EC FP7 QualityNano [INFRA-2010-1.131], contract no: 214547-2, EC FP7 NANoREG, [NMP.2012.1.3-3], contract no: 310584, EC FP7 NanoTOES [PITN-GA-2010-264506], EC FP7-PEOPLE-IEF Marie Curie grant [PIEF-GA-252858] and by NILU internal projects 106179 and 106170. The work by UH Bristol was carried out with the support of the Bristol Centre for Nanoscience and Quantum Information, University of Bristol.

References

Aranda A, Sequedo L, Tolosa L, Quintas G, Burello E, Castell JV, Gombau L. 2013. Dichloro-dihydro-fluorescein diacetate (DCFH-DA) assay: a quantitative method for oxidative stress assessment of nanoparticle-treated cells. Toxicol In vitro 27:954–63.

Berry MN, Friend DS. 1969. High yield preparation of isolated rat liver parenchymal cells. J Cell Biol 43:506–20.

Bhabra G, Sood A, Fisher B, Cartwright L, Saunders M, Evans WH, et al. 2009. Nanoparticles can cause DNA damage across a cellular barrier. Nature Nanotech 4:876–83.

Bright J, Aylott M, Bate S, Geys H, Jarvis P, Saul J, Vonk R. 2011. Recommendations on the statistical analysis of the Comet assay. Pharm Stat 10:485–93.

Brunauer S, Emmett PH, Teller E. 1938. Adsorption of gases in multimolecular layers. Am Chem Soc 60:309–19.

Cartwright L, Poulsen MS, Nielsen HM, Pojana G, Knudsen LE, Saunders M. 2012. In vitro placental model optimization for nanoparticle transport studies. Int J Nanomed 7:497–510.

Cengelli F, Maysinger D, Tschudi-Monnet F, Montet X, Corot C, Petri-Fink A, et al. 2006. Interaction of functionalized superparamagnetic iron oxide nanoparticles with brain structures. J Pharmacol Exp Ther 318:108–16.

Collins AR, Dusinska M, Franklin M, Somorovská M, Petrovská H, Duthie S, et al. 1997. Comet assay in human biomonitoring studies: reliability, validation, and application. Environ Mol Mutag 30:139–46.

Collins AR. 2004. The comet assay for DNA damage and repair: principles, applications, and limitations. Mol Biotechnol 26:249–61.

Correia Carreira S, Walker L, Paul K, Saunders M. 2015. The toxicity, transport and uptake of nanoparticles in the in vitro BeWo b30 placental cell barrier model used within NanoTEST. Nanotoxicology 9(S1):66–78.

Doak SH, Manshian B, Jenkins GJS, Singh N. 2012. In vitro genotoxicity testing strategy for nanomaterials and the adaptation of current OECD guidelines. Mutat Res 745:104–11.

Dodd NJ, Jha AN. 2009. Titanium dioxide induced cell damage: a proposed role of the carboxyl radical. Mutat Res 660:79–82.

Donaldson K, Poland CA, Schins RPF. 2010. Possible genotoxic mechanisms of nanoparticles: criteria for improved test strategies. Nanotoxicology 4:414–20.

Dusinska M, Collins AR. 1996. Detection of oxidised purines and UV-induced photoproducts in DNA, by inclusion of lesion-specific enzymes in the comet assay (single cell gell electrophoresis). ATLA 24:405–11.

Dusinska M, Collins AR. 2008. The Comet assay in human biomonitoring: gene-environment interactions. Mutagenesis 23:191–205.

Dusinska M, Fjellsbø LM, Magdolenova Z, Ravnum S, Rinna A, Runden-Pran E. 2011. Safety of Nanomaterial in Nanomedicine. In: Hunter RJ & Preedy VR, eds. Nanomedicine in Health and Disease. Enfield, NH: Science Publishers Jersey, 203–26.

Dusinska M, Magdolenova Z, Fjellsbø LM. 2013. Toxicological aspects for nanomaterial in humans. In: Oupicky D & Ogris M, eds. Nanotechnology, for Nucleic Acid Delivery' Methods in Molecular Biology. New York: Humana Press, 948:1–12.

Dusinska M, Rundén-Pran E, Carreira SC, Saunders M. 2012. In vitro and in vivo toxicity test methods. Chapter 4. Critical evaluation of toxicity tests. In: Fadeel B, Pietroiusti A & Shvedova A, eds. Adverse Effects of Engineered Nanomaterials: Exposure, Toxicology and Impact on Human Health. New York: Elsevier, 63–84.

Gómez-Lechón MJ, Castell JV. 1998. Primary culture of human hepatocytes. In: Griffiths JB, Doyle A & Newell DG, eds. Cell and Tissue Culture: Laboratory Procedures. England: John Wiley & Sons Ltd. Baffins Lane, 15.1–15.7.

Guadagnini R, HalamodaKenzaoui B, Cartwright L, Pojana G, Magdolenova Z, Bilanicova D, et al. 2015a. Toxicity screenings of nanomaterials: challenges due to interference with assay processes and components of classic in vitro tests. Nanotoxicology 9(S1):13–24.

Guadagnini R, Moreau K, Hussain S, Marano F, Boland S. 2015b. Toxicity evaluation of engineered nanoparticles for medical applications for the pulmonary system. Nanotoxicology 9(S1):25–32.

Halamoda Kenzaoui B, Chapuis Bernasconi C, Guney-Ayra S, Juillerat-Jeanneret L. 2012a. Induction of oxidative stress, lysosome activation and autophagy by nanoparticles in human brain endothelial cells. Biochem J 441:813–21.

Halamoda Kenzaoui B, Chapuis Bernasconi C, Hofmann H, Juillerat-Jeanneret L. 2012b. Evaluation of uptake and transport of ultrasmall superparamagnetic iron oxide nanoparticles by human brain-derived endothelial cells. Nanomedicine 7:39–53.

Harris G, Palosaari T, Magdolenova Z, Mennecozzi M, Gineste JM, Saavedra L, et al. 2015. Iron oxide nanoparticle toxicity testing using high throughput analysis and high content imaging. Nanotoxicology 9(S1):87–94.

Jones HN, Ashworth CJ, Page KR, McArdle HJ. 2006. Cortisol stimulates system A amino acid transport and SNAT2 expression in a human placental cell line (BeWo). Am J Physiol Endocrinol Metab 291: E596–603.

Juillerat L, Dusinska M, Fjellesbo LM, Collins AR, Handy R, Riediker M. 2015. Biological impact assessment of nanomaterial used in nanomedicine. Introduction to the NanoTEST project. Nanotoxicology 9(S1):5–12.

Kain J, Karlsson HL, Möller L. 2012. DNA damage induced by micro- and nanoparticle-interaction with FPG influences the detection of DNA oxidation in the comet assay. Mutagenesis 27:491–500.

Karlsson HL. 2010. The comet assay in nanotoxicology research. Anal Bioanal Chem 398:651–66.

Kazimirova A, Magdolenova Z, Barancokova M, Staruchova M, Volkovova K, Dusinska M. 2012. Genotoxicity testing of PLGA-PEO nanoparticles in TK6 cells by the comet assay and the cytokinesis-block micronucleus assay. Mutat Res 748:42–7.

Kroll A, Pillukat MH, Hahn D, Schnekenburger J. 2012. Interference of engineered nanoparticles with in vitro toxicity assays. Arch Toxicol 86: 1123–36.

Landsiedel R, Kapp MD, Schulz M, Wiench K, Oesch F. 2009. Genotoxicity investigations on nanomaterials: methods, preparation and characterization of test material, potential artifacts and limitations – many questions, some answers. Mutat Res 681:241–458.

Magdolenova Z, Bilaničová D, Pojana G, Fjellsbø LM, Hudecova A, Hasplova K, et al. 2012b. Impact of agglomeration and different dispersions of titanium dioxide nanoparticles on the human related in vitro cytotoxicity and genotoxicity. J Environ Monit 14: 455–64.

Magdolenova Z, Collins AR, Kumar A, Dhawan A, Stone V, Dusinska M. 2014. Mechanisms of genotoxicity. Review of recent in vitro and in vivo studies with engineered nanoparticles. Nanotoxicology 8: 233–78.

Magdolenova Z, Drlickova M, Henjum K, Rundén-Pran E, Tulinska J, Bilanicova D, et al. 2015. Coating-dependent induction of cytotoxicity and genotoxicity of iron oxide nanoparticles. Nanotoxicology 9(S1):44–56.

Magdolenova Z, Lorenzo Y, Collins A, Dusinska M. 2012a. Can standard genotoxicity tests be applied to nanoparticles? J Toxicol Environ Health, part A 75:800–6.

Rajapakse K, Drobne D, Kastelec D, Marinsek-Logar R. 2013. Experimental evidence of false-positive Comet test results due to TiO2 particle- assay interactions. Nanotoxicology 7:1043–51.

Reeves JF, Davies SJ, Dodd NJ, Jha AN. 2008. Hydroxyl radicals (*OH) are associated with titanium dioxide (TiO(2)) nanoparticle-induced cytotoxicity and oxidative DNA damage in fish cells. Mutat Res 640: 113–22.

Sood A, Salih S, Roh D, Lacharme-Lora M, Parry M, Hardiman B, et al. 2011. Signalling of DNA damage and cytokines across cell barriers exposed to nanoparticles depends on the barrier thickness. Nature Nanotech 6:824–33.

Stone V, Johnston H, Schins RPF. 2009. Development of in vitro systems for nanotoxicology: methodological considerations. Crit Rev Toxicol 39:613–26.

Titos E, Chiang N, Serhan CN, Romano M, Gaya J, Pueyo G, Clària J. 1999. Hepatocytes are a rich source of novel aspirin-triggered 15-epi-lipoxin A4. Am J Physiol 277:C870–7.

Tulinska J, Kazimirova A, Kuricova M, Barancokova M, Liskova A, Neubauerova E, et al. 2015. Immunotoxicity and genotoxicity testing of PLGA-PEO nanoparticles in human blood cell model. Nanotoxicology 9(S1):33–43.

Vevers WF, Jha AN. 2008. Genotoxic and cytotoxic potential of titanium dioxide (TiO2) nanoparticles on fish cells in vitro. Ecotoxicology 17: 410–20.

VSN International. 2011. GenStat for Windows. 14th ed. Hemel Hempstead, UK: VSN International.