Overestimation of nanoparticles-induced DNA damage determined by the comet assay

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Abstract

The increasing use of engineered nanoparticles (NPs) in a wide range of commercial products raises concern about the possible risks that NPs pose to human health. Many aspects of the interaction between living cells and NPs are still unclear, and a reliable assessment of NP genotoxicity would be important. One of the most common tests used for genotoxicity is the comet assay, a sensitive method measuring DNA damage in individual cells. The assay was originally developed for soluble molecules, but it is also used in the assessment of genotoxicity of NPs. However, concerns have been raised recently about the reliability of this test in the case of NPs, but no conclusive results have been presented. Using nuclei isolated from human epithelial cells incubated with NPs, we obtained clear evidence of overestimation of NP genotoxicity by the comet assay in the case of CeO₂, TiO₂, SiO₂, and polystyrene NPs. Removal of the NPs in the cytoplasm was effective in eliminating this genotoxicity overestimation (ex post damage) and determining the actual damage produced by the NPs during incubation with the cells (ex ante damage). This method could improve significantly the determination of NP genotoxicity in eukaryotic cells.

Introduction

The interaction between nanoparticles (NPs) and biological systems is receiving growing attention due to rising concern about the possible risks to human health from NPs in the environment. The increasing use of NPs in a variety of commercial products and in drug delivery is increasing the level of exposure to these materials (Chithrani & Chan, 2007; Davda & Labhasetwar, 2002; Gao et al., 2005; Kwon et al., 2014; Smolkova et al., 2015; Unfried et al., 2007). It is, therefore, becoming increasingly important to understand whether and how NPs are detrimental to cell physiology, and more specifically, whether NPs can be directly or indirectly considered as genotoxic factors. Many aspects of the interaction between living cells and NPs are still unclear, despite the large number of investigations on this topic (Cha & Myung, 2007; Kagan et al., 2005; McShan et al., 2014; Salata, 2004). However, defining the potential biological risks associated with exposure to NPs is a complex task. NP toxicity cannot be defined using “one-fits-all” criteria because the interaction between NPs and cells depends strongly on several factors, such as (1) NP physicochemical properties (size, surface area, surface charge, composition, coating, and agglomeration state); (2) level of NP modification produced by interaction with the cellular environment (e.g., coating by serum components); and (3) target cell characteristics (e.g., professional phagocytes, such as monocytes, versus non-professional phagocytes, such as fibroblasts) and differentiation status (e.g., monocytes versus macrophages, and normal versus cancer cells).

Many researchers are trying to clarify the potential genotoxicity associated with NPs (Golbamaki et al., 2015). Comet and micronucleus assays and Ames and chromosome aberration tests are the most common methods used for NP genotoxicity testing, with the comet assay being by far the most widely used (Golbamaki et al., 2015). For instance, in the case of metal oxide/silica NPs, among 165 articles published in 1997–2014 and identified using the key word “genotoxicity”, the comet assay appeared in 83% of them. Despite the wide use of the comet assay in determining NP genotoxicity, its industry and regulatory applications are still very limited.

The single-cell gel electrophoresis (SCGE) assay, commonly known as the comet assay, is a highly sensitive method for measuring DNA strand breaks at an individual cell level (Azqueta & Collins, 2013; Collins, 2004; Duthie & Collins, 1997; Ostling & Johanson, 1984). The comet assay is frequently applied to animal cells or disaggregated animal tissues, and variants have been developed to work with plant cells (Koppen & Angelis, 1998).

The comet assay was originally developed to assess the DNA-strand breaks induced by ionizing radiation and later it was modified to measure DNA damage induced by soluble chemicals. Some doubt has been cast over its use in testing NP genotoxicity...
because the NPs remain during the assay and are not removed as in the case of soluble molecules (Karlsson et al., 2015). This led to the need to validate the consistency of the comet assay in testing NP genotoxicity by comparing its results with those obtained through other methods such as the micronucleus assay (Karlsson et al., 2015). As potential causes of artifacts in the comet assay, Karlsson et al. (2015) considered the possibility that NPs present in or in contact with the cells generate false levels of damage by inducing additional breaks via direct, photocatalytic action or by affecting the nucleoid DNA behavior under electrophoresis. Although they concluded that the comet assay can be trusted for assessing NP genotoxicity (and be included in a standard battery of test methods), Karlsson et al. (2004) were unable to rule out the possibility that additional damage can be produced during the assay, and that the presence of NPs within the nucleoid could interfere with DNA migration.

Here, we investigated whether the comet assay overestimate NP genotoxicity. We modified the standard comet assay using isolated nuclei instead of whole cells. We assumed that eliminating the cytoplasm, which contained most of the NPs incorporated by the cell, would enable us to evaluate only the damage produced by the cell, without the interference of the cytoplasmic debris/material, and if adequate, it was centrifuged 

\[ n \approx \frac{C}{C_1 + C_2} \]

\[ MT \text{tyctotoxicity assay} \]

At the end of the exposure period, 0.9 mM MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Sigma-Aldrich, St. Louis, MO) was added to the cells and after 2 h incubation at 37°C, medium was removed and 100 μM DMSO was added to dissolve the blue formazan that had formed. Cell viability was quantified by measuring absorbance at 540 nm using a microplate reader (Bio-Rad, Hercules, CA) and expressed as a percentage of the control group (that was set to 100%).

**Alkaline comet assay (SCGE)**

DNA strand breaks were detected using the method of Singh et al. (1988). HeLa cells were seeded at 104 cells for 24 h in 60-mm Petri dishes, and the day after (at 50% of confluence), cells were treated with NPs. At the end of treatment, cells were harvested through trypan blue and pelleted by centrifugation at 100 g for 3 min at 4°C and washed twice with cold PBS. Comet assay was performed as described by Collins (2004). For the evaluation of DNA damage, each duplicated sample was analyzed blindly, and for each sample, 100 nuclei were selected at random, scored and classified into five classes according to the visual method described by Collins (2004). The score was performed by a single person well-trained in the comet assay visual score classification. The DNA damage was expressed in arbitrary units (a.u.), where the minimum value was 0 (class 0) and the maximum 400 (class 4). NP-independent DNA damage was induced by means of H2O2 applied to the cells for 30 min at 37°C. Results are given as means ± standard error of the mean (SEM) of three independent experiments (n = 3).

**Nuclei purification for SNGE**

Nuclei purification was carried out by combining and modifying the procedures previously described, for other purposes, by Berkowitz et al. (1969) and Muramatsu et al. (1963). At the end of the treatment, Hela cells were harvested by trypsinization and pelleted at 100 g for 3 min at 4°C. All the following steps were done at 4°C. After two washes with ice-cold PBS, the supernatants were discarded and cell pellets washed with Solution A (0.32 M sucrose, 0.002 M MgCl2, 0.001 M potassium phosphate, pH 6.8) at 4 × 105 cells/ml. The same cell/volume ratio was maintained for all the following steps. After centrifugation at 1000 g for 6 min, the cell pellet was transferred to Solution B (0.01 M NaCl, 0.001 M potassium phosphate, pH 6.8) and kept in the same buffer for 15 min to allow the cells to swell without bursting, while the osmotic swelling was monitored under a microscope. The suspension was centrifuged at 800 g for 5 min. The pellet was suspended in Solution C (10 mM HEPES, pH 7.9, 10 mM KCl, 1.5 mM MgCl2, 0.5 mM DTT, and 1% Triton X-100), supplemented with protease inhibitors by adding Complete Protease Inhibitor Cocktail (Roche, Basel, Switzerland) and PMSF (phenylmethylsulfonyl fluoride, Sigma-Aldrich, St. Louis, MO), and transferred to a pre-cooled 7-ml Dounce tissue homogenizer and lysed by 30 strokes with a tight-fitting pestle. The lysate was examined under a microscope to verify whether the majority of the nuclei (>90%) were free of cytoplasmic debris/material, and if adequate, it was centrifuged at 220 g for 5 min and pelleted in Solution D (0.25 M sucrose and 10 mM MgCl2). Finally, nuclei were collected by centrifugation at 1000 g for 5 min and suspended in 150 μl of 1% low-melting-point agarose and processed accordingly to the method described above for SCGE. The DNA damage was evaluated and analyzed using the same procedure described for SCGE.
**Immunofluorescence and light microscopy**

At the end of the treatment, cells incubated with CeO₂ or fluorescent SiO₂ NPs were processed for immunofluorescence as previously described (Gauthier et al., 2007; Oldani et al., 2009; Ricci et al., 2000), Lysosome-specific mouse-monoclonal anti-Lamp1 primary antibody (BD Biosciences, San Jose, CA) and fluorescent secondary antibody Texas-Red-labeled anti-mouse IgG (Jackson Immunoresearch, West Grove, PA) were used. Samples were analyzed with a TCS SP5II confocal laser scanning microscope equipped with PL APO 40×/1.25 NA and 63×/1.40 NA oil-immersion objectives (Leica, Heidelberg, Germany). Images of the NPs during the different stages of the comet assay, as well as images of intact cells and isolated nuclei, were acquired using an Olympus X83 microscope (Olympus Inc., Tokyo, Japan) equipped with an APO 60×/1.42 NA oil-immersion objective. ImageJ software and related plugins (National Institutes of Health, Bethesda, MD) were used for image processing, intracellular NP quantification, and NP co-localization.

**TEM of biological samples**

After incubation with CeO₂ NPs, whole cells or their isolated nuclei were processed for TEM as previously described (Sommi et al., 2013). Samples were analyzed under a Jeol JEM-1200 EX II transmission electron microscope (Jeol, Tokyo, Japan) equipped with an Olympus CCD camera Mega View III (Olympus Inc., Tokyo, Japan).

**Statistical analysis**

Data are presented as mean ± standard error of the mean (SEM) of three independent experiments (n = 3). Statistical differences between the means were determined by performing Student’s t test or one-way analysis of variance (ANOVA), followed by Newman–Keuls’ Q test. Differences were considered statistically significant for p < 0.05. Data expressed as a percentage of control were analyzed after being normalized versus the controls.

**Results**

**Characterization of NPs**

We investigated four different types of NPs that were selected from among the most commonly used in commercial and/or research applications: CeO₂, TiO₂, SiO₂, and PS (Bitar et al., 2012; Colon et al., 2009; De Jong & Borm, 2008; Ekkapongpisit et al., 2012; Korzeniowska et al., 2013; Smijs, 2011; Spulber et al., 2015; Wason & Zhao, 2013; Xia et al., 2008). The NPs were observed by transmission electron microscopy (TEM) (Figure S1), and characterized by TEM and X-ray diffraction (XRD) (Table 1).

CeO₂ NPs presented a wide distribution in nanoparticle diameter that resulted to be centered just above 20 nm. TiO₂ NPs appeared more uniform, with a similar diameter (20.3 ± 2.1 nm by TEM and 21.3 nm by XRD), and a tendency to form loose agglomerates. SiO₂ and PS presented very uniform, spherical nanoparticles, characterized by a diameter of 40.8 and 24 nm, respectively. Limited agglomeration was observed for both NP types. See Supplementary materials for more details.

**Intracellular NP distribution**

The fate of the NPs at the end of incubation was analyzed by confocal microscopy (Figure 1). Exposure to increasing concentrations of NPs produced a parallel increase in the overall number of lysosomes, the intracellular compartment with a key role in degrading and processing exogenous as well as endogenous material; shown here in the case of CeO₂ (Figure 1A) as well as for PS NPs (Figure 2S2). TiO₂ NP effect was analogous to that observed for CeO₂ (data not shown). Fluorescent NPs (SiO₂) demonstrated the co-localization between lysosomes and NPs (Figure 1B). Most of the intracellular NPs were localized within the lysosomes. In response to the increase in NP concentration, there was a parallel increase in the numbers of lysosomes and co-localized NPs (Figure 1B). There was no detectable fluorescent signal, in the case of SiO₂, in interphase/prophase nuclei.

**Evaluation of DNA damage**

Before proceeding with DNA damage evaluation, we ensured that exposure to NPs (CeO₂: 50 and 200 µg/ml; TiO₂: 50 and 200 µg/ml; SiO₂: 50, 200 and 500 µg/ml; and PS: 10, 50 and 100 µg/ml) did not alter the cell viability and so affect the subsequent DNA damage analyses. At the end of the incubation period, the cells were tested for cytotoxicity by MTT assay and there was no large decrease in viability (Figure S3). DNA strand breaks were analyzed by comet assay in HeLa cells incubated with the different NP concentrations. It must be noted that due to the different nature of the NPs, the concentrations able to produce damage resulted to be different in each case. The incubation time was set at 48 h for all the NPs with the exception of PS, which required longer to observe a significant level of damage. In the first part of this study, the comet assay was performed using whole cells, following the standard procedure for eukaryotic cells. In this procedure, NPs that were internalized at the end of the treatment could still remain within the cells throughout the comet assay. The DNA damage was evaluated using the five-classes score (Figure S4). The second part of the analysis was performed using isolated nuclei only. The number of NPs was reduced due to elimination of the cytoplasmic fraction that contained most of the NPs.

**Single-cell gel electrophoresis (SCGE)**

When CeO₂ NPs were incubated with the cells for 48 h, there was a high level of DNA damage with concentrations of 50 and 200 µg/ml (Figure 2A). Similar results were observed for TiO₂ NPs, with both concentrations (Figure 2B). In the case of SiO₂ NPs, there was a slight increase in DNA damage at concentrations of 50 and 200 µg/ml. A medium level of DNA damage was obtained by increasing the concentration to 500 µg/ml (Figure 2C). The situation was different for PS NPs (Figure 2D). Concentrations of 50 and 100 µg/ml gave a low/medium level of damage, while no difference from control was observed at 10 µg/ml NP. These were low levels of damage, which were consistent with the well-known low toxicity associated with this type of NP (Ekkapongpisit et al., 2012). Extending the incubation time to 120 h, the level of DNA damage was slightly increased, with the higher concentrations inducing a medium level of damage. Higher level of DNA damage could not be observed at concentration above 100 µg/ml at both 48 and 120 h.

To understand the behavior and distribution of these NPs, we followed them through different stages of the assay.

| NP type | TEM particle size (nm) | XRD grain size (nm) |
|---------|------------------------|---------------------|
| CeO₂    | 19.8 ± 0.2             | 24.2                |
| TiO₂    | 20.3 ± 2.1             | 21.3                |
| SiO₂    | 40.8 ± 1.9             | n/a                 |
| PS      | 24.0 ± 2.8             | n/a                 |

TEM results are given as mean ± SEM.
(agarose embedding, membrane lysis, alkaline incubation, and electrophoresis). This evolution is shown in Figure 3 for the fluorescent NPs (SiO$_2$ and PS). In the early stages of the process, the NPs were localized in close proximity to the nucleus (Figure 3A and E), but after membrane lysis (Figure 3B and F) the NPs, released from their membrane-enveloped compartments, surrounded naked DNA in a random manner. After an additional 40 min in the electrophoretic solution (Figure 3C and G), the NPs became more diffuse, and after electrophoresis (Figure 3D–D2 and H–H2), their distribution changed significantly. They became more concentrated on one side of the nucleoid with a tendency to spread towards the cathode, producing a sort of diffuse tail, mimicking the DNA tail.

Single-nucleus gel electrophoresis (SNGE)

After incubation with NPs, the cell cytosolic fraction, containing most of the internalized NPs, was removed and the nuclei purified. Even minimal cell mishandling can easily affect the comet assay; therefore, the procedure for isolation of nuclei was optimized to exclude any procedure-related damage and ensure consistent and reproducible results. Using untreated (not exposed to NPs) HeLa cells, the procedure was adjusted in all its steps (type of buffers, time of cell swelling, and numbers of strokes) to obtain isolated nuclei without any sign of DNA damage. Nuclei considered suitable for SNGE had to be without any cytoplasmic debris. During the procedure setup, fluorescent NPs were used to help monitor removal of the cytoplasmic material. Contaminated nuclei were demonstrated by the presence of NPs, while the absence of any detectable NPs was considered an indication of good nuclear isolation (Figure 4A–C). TEM confirmed, in the case of CeO$_2$ NPs, that the isolation procedure was successful in obtaining purified nuclei. All the NPs present in the cytoplasm appeared to be removed, although preserving those already present inside the nuclei (Figure 4D–G).

To establish whether the purified nuclei were suitable for genotoxicity evaluation, the comet assay was performed on nuclei obtained from control cells, and the results were compared with those using the standard procedure on control whole cells. The results obtained with the two procedures were comparable, showing no sign of DNA damage (Figure 5A).

To evaluate further the method’s reliability, we used H$_2$O$_2$-treated cells as a positive control, assuming that the DNA damage induced by this soluble molecule had no ex post effect. Cells were treated with H$_2$O$_2$ (up to 200 μM) for 30 min at 37°C and the isolated nuclei were used to perform the
comet assay. While the highest H₂O₂ concentration (i.e., 200 μM) induced high DNA damage with both the SCGE and SNGE procedure tested, by using non-saturating H₂O₂ concentrations (i.e., 50 and 100 μM), we observed the same class of damage for both the procedures, the SNGE one showing an increased level of about 30–44% compared with SCGE (Figure 5A).

Once it was verified that no DNA damage was produced by the nuclear purification procedure, we defined a classification for DNA damage in the isolated nuclei. This was done to exclude the possibility that the isolated nuclei could produce a different comet appearance (especially in the case of intermediate levels of DNA damage, i.e., classes 2 and 3), making it difficult to compare the SNGE and SCGE results.

Purified nuclei from cells incubated with increasing H₂O₂ concentrations were analyzed by a person well-trained in the comet assay visual score classification. For each condition, 100 nucleoids in duplicate were scored and classified into five classes indicating the level of damage (Figure 5B). No aberrant tails or nucleoids were noted during this analysis, and subdivision into classes for SNGE (Figure 5B) was similar to the five classes commonly used for SCGE (Figure S4). In particular, SNGE class 1 showed a distinctive pronounced tail compared with SCGE class 1, which showed instead a small radial DNA distribution around the head.

Figure 2. SCGE of HeLa cells incubated with CeO₂ (A), TiO₂ (B), SiO₂ (C), and PS (D) NPs. 0, untreated cells. H₂O₂, cells treated for 30 min at 37°C with 200 μM were used as a positive control. The DNA damage is expressed in a.u., where the minimum value is 0 (class 0) and the maximum 400 (class 4). Results are given as means values ± SEM of three independent experiments (n = 3). *p<0.05, **p<0.01, ***p<0.001 versus each respective control.

Figure 3. NP distribution during the comet assay procedure. HeLa cells, previously incubated with fluorescent SiO₂ (500 μg/ml) or PS (100 μg/ml) NPs, were trypsinized and embedded in agarose (A and E), kept in alkaline lysis for 90 min (B and F), then 40 min in electrophoresis solution (C and G), and finally subjected to electrophoresis (D and H). Green, NP; blue, DNA; gray, DNA in D, D1, H and H1. Bar, 5 μm.
the head of the comet. Another difference was in the dimension/shape of class 2/3 tails, which in SNGE, appeared wider than the same class in SCGE. The subdivision into classes (Figure 5B) was then used for all the subsequent tests.

The results of the genotoxicity tests for the different NP types obtained with or without nuclear isolation (SNGE versus SCGE) are compared in Figure 6. With the exception of PS NPs, for which a 120 h-incubation period was used for its slight increment compared with 48 h incubation and a possible cumulative effect, all the data relate to an incubation time of 48 h. In the case of CeO₂ (Figure 6A), the reduction obtained by SNGE was significant, resulting in a shift of class for both concentrations of 50 and 200 μg/ml, going from class 3 for SCGE to class 2 for SNGE. A similar result was observed for 50 and 200 μg/ml TiO₂ NP (Figure 6B), where an evident reduction was obtained by SNGE, resulting in a shift from class 2/3 for SCGE, to class 1/2 for SNGE. For SiO₂ NPs (Figure 6C), a reduction in DNA damage from class 2 to class 1 was observed only for the highest NP concentration (500 μg/ml). No significant reduction was demonstrated for 50 and 200 μg/ml, at which concentration, the DNA damage detected by SCGE was already low (class 1). For PS NPs, the level of damage detected by SNGE after 120 h incubation was significantly reduced in comparison with the damage evaluated by SCGE (Figure 6D). In the case of 50 and 100 μg/ml, a shift from class 2 (SCGE) to class 1 (SNGE) was observed.

Our results suggest that when the DNA damage evaluated by SCGE is already low, elimination of the cytoplasm does not markedly modify the results. In contrast, when the damage evaluated by SCGE is elevated (medium or higher), elimination of the cytoplasmic fraction results in a significantly lower level of damage. The detected DNA damage reduction would be even lower considering that in control cells, SNGE gave rise to higher score than SCGE, as for H₂O₂-induced DNA damage where there was no reduction by the elimination of the cytosol but even an increase (Figure 6, gray circles). Our observations thus support the conclusion that the presence of free NPs during the comet assay may produce interference that seems to be dose dependent.

Discussion

The comet assay is the most common method for evaluating DNA damage in single eukaryotic cells or in disaggregated tissues (Azqueta & Collins, 2013; Collins, 2004). This method is widely used in genotoxicity testing, human biomonitoring, and in ecogenotoxicology (Azqueta & Collins, 2013). Although the comet assay has been extensively used to investigate the possible relationship between NPs and DNA damage, the literature shows an increasing skepticism in this regard, due to the discrepancy with the results obtained using other methods (Karlsson, 2010; Lin et al., 2009; Rajapakse et al., 2013). Although recently it has been suggested that the comet assay is reliable for assessment of NP genotoxicity (Golbamaki et al., 2015; Karlsson, 2010; Karlsson et al., 2015), there is still not enough experimental evidence to support this conclusion, and the overall problem of NPs interfering with the assay outcome has been specifically investigated by only few studies (Gerloff et al., 2009; Karlsson, 2010; Lin et al., 2009; Magdolenova et al., 2012; Petersen & Nelson, 2010; Rajapakse et al., 2013).

One concern regarding the use of the comet assay for assessing NP genotoxicity is due to the localization of the NPs during the test itself. At the end of the procedure, NPs are usually localized
in large numbers in the nucleoid or the head of the comet (Karlsson, 2010). In contrast, TEM of cells previously incubated with NPs does not show many NPs in the nucleus (Karlsson, 2010; present study). This difference in distribution might derive from the assay procedure. After the cells are processed and embedded in agarose during the comet assay, they assume a spherical shape, with all their components localized in close proximity to the nucleus, including the lysosomes, which contain most of the internalized NPs. After standard alkaline treatment and degradation of the membranes, the NPs are not eliminated or diffused but they persist around what has become bare DNA. Considering the high chemical activity usually shown by the NPs, this co-localization suggests a strong interaction between NPs and bare DNA. Indirect proof of such a strong interaction is given by the evolution in the NP distribution observed during the comet assay. The NPs are tightly distributed around the nucleus/nucleoid before being subjected to electrophoresis, but their distribution around the nucleoid is significantly influenced by the electric field applied during the test (Figure 3). After electrophoresis, the NPs drift towards the cathode, producing a tail that mimics the DNA tail. In contrast, if NPs alone (without any cellular material) are embedded in the gel and exposed to the same electrophoretic cycle used in the comet assay, no evidence of drifting is observed (Figure S5). This indicates that the observed NP redistribution derives from a strong interaction between the NPs and DNA, with the former following the movements of the DNA during electrophoresis. This interaction might take place once the NPs are released from membrane-enveloped compartments in close proximity to the nucleoid in the preliminary stages of the comet assay.

The NP–DNA binding process has been investigated previously and evidences of a strong interaction have been presented (An & Jin, 2012; Chen et al., 2010; Goodman et al., 2006). The evidence of a strong interaction between NP and DNA supports the possibility that the NPs interact with DNA in the nucleoid and interfere with the comet assay. In the present study, we did obtain evidence for significant interference by NPs with the outcome of the comet assay. We reached this conclusion by comparing the DNA damage monitored using the whole cells (SCGE) and the purified nuclei (SNGE) derived from cells previously incubated with NPs. It is difficult to establish whether NPs directly induce damage by binding to and fragmenting DNA, or if they simply modify the tail formation, producing a longer tail by interfering with the DNA charges. Railsback et al. (2012) have demonstrated in silico that even weakly charged gold NPs may compromise the structural integrity of dsDNA by promoting the
formation of ssDNA. They found that, for high concentrations (≥3 particles), NPs attached to the DNA could bend and separate the DNA strands.

To remove the interference of the cytoplasmic NPs during the comet assay, we investigated the possibility of performing the comet test only on the purified nuclei fraction, following a procedure that is often used in genotoxicity tests in plant cells (Angelis et al., 1999; Koppen & Angelis, 1998). The use of purified nuclei from eukaryotic cells allowed us to remove all the NPs that were not already incorporated in the nuclei before the comet assay was performed. This enabled us to discriminate between the ex ante damage (caused during the true NP–cell interaction) and the ex post damage (artifactually caused during the assay itself).

The NPs used for this study were from among the most commonly used in commercial and/or research applications. CeO2 NPs have received much recent attention for their unique regenerative antioxidant properties (Colon et al., 2009; Spulber et al., 2015; Wason & Zhao, 2013; Xia et al., 2008); TiO2 NPs are commonly included in the formulation of several industrial products (Smij, 2011); SiO2 NPs have wide application in several areas of nanotechnology (Bitar et al., 2012; Korzeniowska et al., 2013); and PS NPs have been largely investigated for possible application in drugs delivery (De Jong & Borm, 2008; Ekkapongpisit et al., 2012).

NP internalization is a time- and concentration-dependent process: the longer the time of contact, the more NPs are internalized (Sommi, personal observation). DNA damage evaluated by the standard comet procedure/SCGE shows a similar trend, as higher NP concentrations and longer exposure induce more damage. This may be due only to ex ante DNA damage, but it could also be partially caused by an ex post effect, produced by NPs accessing the DNA during the assay. For all the NPs we investigated, removal of the cytoplasm was more effective in reducing the DNA damage the more that NPs were internalized by the cells, often producing a shift in the level of damage toward the lower classes. This observation supports the conclusion that elimination of the cytoplasmic fraction significantly lowers the level of DNA damage for all the NPs. It must also be inferred that elimination of the cytoplasmic NPs produces comet assay results that are more reliable, approximating better the real level of genotoxicity produced by the actual cell–NP interaction before the comet assay is performed.

The possibility of artifacts resulting from direct interference of the NPs with genomic material during the comet assay has been raised previously (Gerloff et al., 2009; Karlsson, 2010; Karlsson et al., 2015; Lin et al., 2009; Magdolenova et al., 2012; Petersen & Nelson, 2010; Petersen et al., 2014; Rajapakse et al., 2013). Magdolenova et al. (2012) added NPs directly to the cells just before embedding them into agarose. With the exception of Fe3O4, no increment in DNA strands breaks due by the presence of the NPs was observed, consenting the authors to exclude any possible interference of NPs with the comet assay. This approach, however, did not allow any NPs cellular internalization and does not exclude the possibility that NP already internalized can exert any interference with the assay. Using a different kind of NPs, proven to cause substantial DNA damage, Karlsson et al. (2015) observed that the DNA damage evaluated by comet assay was not related to the amount of NPs present on the cell surface. In contrast, a substantial amount of DNA damage was observed in cells that were only exposed during the last step of the assay performance (Karlsson et al., 2015). This observation proved that DNA-damaging particles present during the assay can cause additional DNA damage. The authors, however, excluded that under normal experimental condition the intracellular NPs present during the assay can account for any additional damage, considering that they represent only a small percentage of the overall NPs. These observations partially agree with our finding that the presence of NPs during the comet assay could give rise to an overestimation of the damage. In our case, however, by

Figure 6. NP genotoxicity evaluated by SNGE. (A–D) Comparison between SNGE (black circles, solid line) and SCGE (white circles, dotted line) for cells previously incubated with CeO2 (A), TiO2 (B), SiO2 (C), and PS (D) NPs. An additional curve for SNGE (gray circles, solid line) shows an even lower level of damage when SNGE-line is normalized versus the SCGE-line at 0 μM point. Results are given as means values ± SEM of three independent experiments (n = 3). *p < 0.05, **p < 0.01 versus each respective SCGE point.
eliminating the NPs accumulated in the cytoplasm, we demonstrate that even those NPs accumulated inside the cells could interfere, especially in the case of the higher concentrations.

Lin et al. (2009) observed that the genotoxicity evaluated for germanium by comet assay was not confirmed by a comparable test, considering the level of H2AX histone phosphorylation (\(\gamma\)-H2AX) as an indication of DNA damage. To explain this discrepancy, the authors speculated that germanium NPs could ‘‘tear down’’ the DNA of lysed cells during electrophoresis, thus forming significant comet tails and false-positive results. Further evidence of possible artifacts due to the presence of NPs during the comet assay comes from the observation that different light conditions may influence the assay when performed in the presence of photocatalytic NPs, like TiO\(_2\), which can induce additional DNA breaks (Gerloff et al., 2009). A possible approach for evaluating the occurrence of such interference has been proposed recently by Rajapakse et al. (2013). They used *Tetrahymena thermophila*, a unicellular ciliated protozoan, to test TiO\(_2\) genotoxicity by comet assay. To identify NP comet assay interference, they evaluated the DNA damage by performing the comet assay on control cells embedded in agarose, where, after alkaline lysis, the nuclei were exposed to the NPs directly applied to the gel (acellular comet assay) and then subjected to electrophoresis. This acellular approach, although useful in obtaining information on the possible interference of NPs in the comet assay, does not offer all the advantages provided by the SNGE such as complete comparability with SCGE. In fact, the acellular test is difficult to compare directly with the whole cell comet assay. In contrast, by comparing the results of SNGE and SCGE, it is possible to obtain direct information on both the *ex ante* effect and *ex post* interference, evaluating at the same time, whether the NPs under investigation are genotoxic and/or interfere with the assay. Not having the NPs present during the assay favors a more reliable result, which does not include the *ex post* damage. SNGE yields information only about the real NP genotoxicity exerted by the NPs, due to direct interaction with DNA or by indirect effects caused through reactive oxygen species production or DNAase released by damaged lysosomes. This type of comparison would be otherwise difficult to perform between the whole cells and the acellular test because the NP concentration in the already embedded acellular system is different from the intracellular concentration, making it difficult to subtract the interference effect and obtain clean data about the real NP genotoxicity. It must also be considered that the NPs accumulated in the lysosomes can reach high local concentrations, which could be difficult to match if NPs are applied directly on the gel. Besides, the proximity of the NPs to the nuclei would be difficult to reproduce.

The recent review by Karlsson et al. (2015) discussed the possibility of an interference of NPs with the comet assay. The authors, while excluding the existence of any additional damage induced during the assay under normal experimental conditions, left open some questions regarding this topic and recognize the general lack of experimental evidences. In this study, we have been able to demonstrate that interference is indeed possible at least for the NP and the concentrations we have investigated.

**Conclusion**

We demonstrate the existence of a clear interference between CeO\(_2\) and TiO\(_2\) NPs and the outcome of the comet assay. A less pronounced effect has been observed in the case of SiO\(_2\) and PS NPs. To eliminate this interference, we propose a new approach consisting of performing the comet assay on nuclei alone, isolated from cells previously treated with NPs. By eliminating the cytoplasmic fraction of the treated cells, we demonstrated that most of the NPs were not present during the assay, and the level of DNA damage was reduced, yielding a result that approximated better the real level of genotoxicity induced by the NPs.

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**Declaration of interest**

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