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Contribution of ionic silver to genotoxic potential of nanosilver in human liver HepG2 and colon Caco2 cells evaluated by the cytokinesis-block micronucleus assay

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ABSTRACT: Extensive human exposure to food- and cosmetics-related consumer products containing nanosilver is of public concern because of the lack of information about their safety. Genotoxicity is an important endpoint for the safety and health hazard assessment of regulated products including nanomaterials. The *in vitro* cytokinesis-block micronucleus (CBMN) assay is a very useful test for predictive genotoxicity testing. Recently, we have reported the genotoxicity of 20 nm nanosilver in human liver HepG2 and colon Caco2 cells evaluated using the CBMN assay. The objective of our present study was three-fold: (i) to evaluate if HepG2 and Caco2 cells are valuable *in vitro* models for rapid genotoxicity screening of nanosilver; (ii) to test the hypothesis that the nanoparticle size and cell types are critical determinants of its genotoxicity; and (iii) to determine if ionic silver contributes to the nanosilver genotoxicity. With these objectives in mind, we evaluated the genotoxic potential of 50 nm nanosilver of the same shape, composition, surface charge, obtained from the same commercial source, under the same experimental conditions and the same genotoxic CBMN endpoint used for the previously tested 20 nm silver. The ionic silver (silver acetate) was also evaluated under the same conditions. Results of our study show that up to the concentrations tested in these cell types, the smaller (20 nm) nanosilver induces micronucleus formation in both the cell types but the larger (50 nm) nanosilver and the ionic silver provide a much weaker response compared with controls under the same conditions. Published 2016. This article is a U.S. Government work and is in the public domain in the USA.

Keywords: nanosilver; silver nanoparticles; nanoparticles; genotoxicity; micronucleus; HepG2; Caco2; cytokinesis-block micronucleus; *in vitro* micronucleus

Introduction

Nanosilver is one of the most commonly used nanomaterials in food- and cosmetics-related consumer products (Morris, 2011; Rashidi and Khosravi-Darani, 2011) because of its broad spectrum of antibacterial (Lok *et al*., 2006) and antifungal (Sondi and Salopek-Sondi, 2004) properties. Extensive human exposure to these products is of public concern owing to a lack of information about their safety. Genotoxicity is an important toxicity endpoint for the safety and health hazard assessment (ICH, 2011) of regulated products including nanomaterials. Therefore, rapid screening of the genotoxic potential of nanosilver is necessary to ensure consumer safety.

The use of animals for safety assessment is costly and time-consuming. Therefore, the search for suitable alternative *in vitro* models to accurately predict *in vivo* toxicity is necessary and important. The National Research Council (NRC) has recognized the importance of cell cultures, especially of human origin, as *in vitro* models for toxicity testing for safety assessment (NRC, 2007). Therefore, various *in vitro* cell culture models need to be evaluated to predict the toxicity and generate reliable and reproducible mechanistic information that may be used for safety evaluation (Meek and Doull, 2009).

The gastrointestinal tract is an important route of exposure for food-related nanosilver (Kim *et al*., 2008). Information on human exposure to nanosilver through the gastrointestinal tract and its subsequent systemic absorption is limited at the present time. Translocation of nanosilver from blood to the liver has been demonstrated in the rat (Sung *et al*., 2009; Dziendzikowska *et al*., 2012) and mouse (Xue *et al*., 2012). Numerous reports demonstrate that the human liver HepG2 and human colon Caco2 cells, representing the liver and gastrointestinal tract, respectively, are valuable *in vitro* cell culture models for toxicity screening of test agents of interest. Both the HepG2 (Schoonen *et al*., 2005a, 2005b; O'Brien *et al*., 2006; Xu *et al*., 2008; Jennen *et al*., 2010;) and Caco2 (Brandon *et al*., 2006; Bouwmeester *et al*., 2011; Gerloff *et al*., 2009, 2013) cells have been extensively used in toxicity studies for the screening of classic toxicants. Therefore, we evaluated these two widely used human *in vitro* cell culture models, Caco2 and HepG2 cells,

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representing gastrointestinal absorption and subsequent liver exposure, to assess the genotoxic potential of nanosilver (Sahu *et al*., 2014b).

Genotoxicity, the capacity of an agent to cause damage in the genetic material directly or indirectly, is an important endpoint for the safety and hazard assessment of regulated products including nanomaterials. However, no single test is available to detect all types of genotoxic effects. Therefore, a battery of tests is recommended by the internationally harmonized guidance (ICH 2008) for hazard identification of the broadest set of genotoxic agents. The *in vitro* cytokinesis-blocked micronucleus (CBMN) assay is a very useful test for predictive genotoxicity screening (Fenech, 2000, 2007; OECD, 2014). This assay standardized under the OECD guidelines (OECD, 2014) is widely used for genotoxicity screening of potential genotoxicants.

The mechanism of nanosilver genotoxicity is unknown. Information available in the literature is contradictory. There is an ongoing debate on the contribution of ionic silver (Ag^+) to nanosilver (AgNP) toxicity. Some studies show that the silver nanoparticles themselves are responsible for their toxicity different from the actions of the ionic silver (Powers *et al*., 2011). Some studies report that AgNP has the potential for the intracellular release of silver ion (Ag^+) leading to toxicity (Ho *et al*., 2010; Jiang *et al*., 2015; Lubick, 2008). Some reports indicate that AgNP and Ag^+ have different intrinsic properties and different mechanisms of action (Jiao *et al*., 2014). Some reports have shown that the combination of AgNP and Ag^+ is more toxic than either form of silver alone (Bae *et al*., 2010; Sotiriou and Pratsinis, 2010). Therefore, Sotiriou and Pratsinis (2010) have proposed multiple modes of action for AgNP and different modes of action operate under different conditions depending on the size of the AgNP (smaller or larger than 10 nm in diameter).

Recently, we have demonstrated that the human liver HepG2 and colon Caco2 cell cultures, which have been widely used for decades in toxicity evaluation of conventional chemical toxicants, are excellent *in vitro* models for predictive genotoxicity screening of 20 nm nanosilver evaluated by the CBMN assay (Sahu *et al*., 2014b). The purpose of the present study was threefold: (i) to evaluate if HepG2 and Caco2 cells are valuable *in vitro* models for rapid genotoxicity screening of nanosilver, (ii) to test the hypothesis that the nanoparticle size and cell types are critical determinants of its genotoxicity, and (iii) to determine if ionic silver (silver acetate as a representative ionic silver) contributes to the genotoxic potential of nanosilver, using the same *in vitro* model, CBMN genotoxic endpoint and experimental conditions as the 20 nm silver (Sahu *et al*., 2014b).

Materials and methods

Materials

The 50 nm BioPure® silver nanoparticle citrate solution was purchased from the same commercial source as the 20 nm silver (nanoComposix, San Diego, CA, USA). Human hepatoblastoma HepG2 cells (ATCC HB-8065) and human colon carcinoma Caco2 cells (ATCC HTB-37) were obtained from the American Type Culture Collection (ATCC), Manassas, VA, USA. Deep-frozen vials of stock cells were routinely stored in a liquid nitrogen freezer. Dulbecco's modified Eagle's medium (DMEM) GlutaMax, Hanks' balanced salt solution (HBSS), HEPES, phosphate-buffered saline (PBS), trypsin-EDTA solution and 0.4% trypan blue solution were purchased from Invitrogen Corporation (Grand Island, NY, USA). Fetal bovine serum (FBS) was purchased from the Hyclone Labs (Logan, UT, USA). The sterile nonpyrogenic polystyrene cell culture flasks and plates were purchased from Corning (Corning, NY, USA) and Becton-Dickinson (Franklin Lakes, NJ, USA), respectively. ReagentPlus grade silver acetate, etoposide, acridine orange and all other chemicals and reagents were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). Dimethyl sulfoxide (DMSO) was purchased from Baker (Phillipsburg, NJ, USA).

Methods

Characterization of 50 nm nanosilver

The silver nanoparticles were characterized by dynamic light scattering (DLS), transmission electron microscopy (TEM) and inductively coupled plasma–mass spectrometry (ICP–MS) analysis as described previously (Sahu *et al*., 2014a,b). The stock solution of the nanosilver in aqueous 2 mM citrate was stored at 4 °C in small aliquots. The desired concentrations of silver nanoparticles for cell exposures were prepared fresh by diluting the stock solution with the cell culture medium just before the experiment (Sahu *et al*., 2014a, b).

Cell culture

Human hepatoblastoma HepG2 cells and human colon carcinoma Caco2 cells were stored routinely in small aliquots in liquid nitrogen and the experimental cultures were prepared from the frozen stock cells and always kept in a subconfluent state (Sahu *et al*., 2014a, b). The cells were cultured in Dulbecco's modified Eagle's medium (DMEM) with GlutaMax containing 1.5% glucose and supplemented with 10% FBS, 1% MEM non-essential amino acids and 10 mM HEPES buffer (Sahu *et al*., 2014a, b). The cells were cultured in a saturating humidified atmosphere of 5% CO_2 in air at 37 °C. The culture medium was changed every 3–4 days.

Silver acetate solution

A stock solution of 1.55 mg silver acetate ml^{-1} (1.0 mg silver ion ml^{-1}) was prepared fresh for each experiment on the day of treatment. The desired concentrations of silver ion for cell exposures were prepared fresh by diluting this stock solution with the cell culture medium just before each experiment.

Evaluation of cytotoxicity

Cells growing exponentially at approximately 70–80% confluence were used in the preparation of seeding culture. On the day of treatment, the dosing solutions of the 50 nm silver and ionic silver were prepared by serial dilutions of the stock solution in the cell culture medium immediately before use as reported for the 20 nm silver (Sahu *et al*., 2014a, b).

The concentrations of the dosing solutions and the time of exposure were selected based on our previous studies (Sahu *et al*., 2014a, b) showing the exposure time- and dose-dependent cytotoxicity and genotoxicity of 20 nm silver in HepG2 and Caco2 cells with HepG2 found to be more sensitive to the nanosilver (Sahu *et al*., 2014a, b). These cells were exposed to the 50 nm nanosilver in a concentration range from 5.0 to 50.0 μg ml–1 and to the ionic silver (silver acetate) in a concentration range

from 0.2 to 3.0 μg ml^{-1} to determine a dose-response for cellular cytotoxicity.

The loss of cell viability, determined by the resazurin (Alamar Blue) assay, was a measure of cytotoxicity (O'Brien *et al.*, 2006; Shao *et al.*, 2008; Chopra *et al.*, 2009; Gaiser *et al.*, 2013; Sahu *et al.*, 2014a, 2014b;). Briefly, the cells seeded in 96-well plates for 24 h were treated with the vehicle control and desired concentrations of the test material. Then they were washed with sterile HBSS and incubated with resazurin (Chopra *et al.*, 2009; Sahu *et al.*, 2014a, b) for 30 min at 37 °C in a plate reader using the Sigma Resazurin Assay kit. The rate of increase of resorufin fluorescence was measured at 545 nm excitation and 590 nm emission.

Evaluation of genotoxicity by the CBMN assay

The genotoxicity of the 50 nm nanosilver and the ionic silver (silver acetate) was evaluated according to the OECD Guideline, 487 (OECD, 2014) and the current literature (Fenech, 2000, 2007) using the CBMN assay with acridine orange staining and fluorescence microscopy as previously described in detail (Sahu *et al.*, 2014b). Also, we followed the recommendations of Elespuru *et al.* (2009) and ICH (2008, 2011) for the use of higher concentrations of potential genotoxicants than recommended by the OECD guidelines to assure the hazard identification required for safety assessment.

Cells growing exponentially at approximately 70–80% confluence were used in the preparation of seeding culture. The cells were washed with Ca- and Mg-free HBSS and dislodged from the culture flask by brief treatment with 0.05% trypsin-EDTA. The single cell suspension in the culture medium was obtained by repeated trituration. Cell counts and cell viability were determined by trypan blue dye exclusion using a hemocytometer. A single cell suspension in the culture medium at a density of 5×10^5 cells ml^{-1} was prepared by serial dilution and the cell suspension was used for seeding cells in two-chambered slides at the desired cell density needed for particular experiments. Cells were then incubated for 24 h at 37 °C in a saturated humidified atmosphere of 5% CO_2 in the air.

On the day of treatment, the dosing solutions of 50 nm silver and ionic silver were prepared by serial dilutions of the stock solution in the cell culture medium immediately before use as reported for the 20 nm silver (Sahu *et al.*, 2014a, b). The cells were washed once with HBSS and the dosing solutions were added to the cells in replicate wells of culture slides.

For genotoxicity evaluation of the 50 nm nanosilver, we followed the OECD guidelines (OECD, 2014) that recommend the highest concentration to be tested in the CBMN assay should produce 55 ± 5% cytotoxicity as well as the recommendations of Elespuru *et al.* (2009) and ICH (2008, 2011) for use of higher concentrations of potential genotoxicants than recommended by the OECD guidelines to assure the hazard identification required for safety assessment. Our preliminary experiment was designed bearing in mind the reports of Karlsson *et al.* (2009), which suggest that all sizes of a nanoparticle with the same chemical composition and same all other factors may not show the same toxicity potential, and that of Sahu *et al.* (2014a, b), which show a concentration-dependent uptake of nanosilver. Therefore, in our first preliminary experiments for the 50 nm nanosilver, we used the test concentration range and all other experimental conditions close to that of 20 nm nanosilver (Sahu *et al.*, 2014b). Based on the level of cytotoxicity of 50 nm nanosilver and our previous results of 20 nM nanosilver (Sahu *et al.*, 2014a, b), our first preliminary CBMN genotoxicity experiment on 50 nm nanosilver was conducted at a concentration range of 0.0 to 20 μg ml^{-1}. We evaluated the genotoxicity of 50 nm nanosilver in HepG2 and Caco2 cells using the same experimental conditions and the genotoxic endpoint (CBMN) as reported previously for the 20 nm nanosilver (Sahu *et al.*, 2014b) according to the OECD guidelines (OECD, 2014). We observed that there was not sufficient cytotoxicity (~50% compared to solvent) and genotoxicity in the first CBMN genotoxicity experiment for the 50-nm nanosilver at the highest dose tested. Under such circumstances, the use of higher concentrations in the CBMN test has been suggested for suspected genotoxins to assure their potential hazard identification (Elespuru *et al.*, 2009; ICH, 2008, 2011). Therefore, we repeated the CBMN test for 50 nm silver in HepG2 and Caco2 cells at the higher concentration range of 10.0 to 100 μg ml^{-1} for 4-h exposure and 2.5 to 25 μg ml^{-1} for 24-h exposure, bearing in mind that the genotoxicity of 20 nm silver was exposure time- and dose-dependent in both the cell types in our previous study (Sahu *et al.*, 2014b). The control cells received an equal volume of the vehicle cell culture medium. Etoposide was used as the positive control (Bryce *et al.*, 2008; Bryce *et al.*, 2010; Fowler *et al.*, 2010; Sahu *et al.*, 2014a, b). The cells were treated for either 4 h or continuously for 24 h at 37 °C in a saturated humidified atmosphere of 5% CO_2 in the air. In the short-term 4-h exposure, the treatment medium was aspirated after 4 h, the cells were washed with Ca- and Mg-free HBSS, re-fed with complete culture medium containing cytochalasin B (3.0 μg ml^{-1}) and incubated at 37 °C in a saturated humidified atmosphere of 5% CO_2 in air for an additional 20 h. For the continuous 24-h exposure, cytochalasin B (3.0 μg ml^{-1}) was added at the beginning of the treatment.

For genotoxicity evaluation of the ionic silver (silver acetate), we also followed the OECD guidelines (OECD, 2014) and the recommendations of Elespuru *et al.* (2009) and ICH (2008, 2011) for the use of higher concentrations of potential genotoxicants than recommended by the OECD guidelines to assure the hazard identification required for safety assessment. We evaluated the genotoxic potential of ionic silver in the concentration range from 0.1 to 5.0 μg ml^{-1}. The lower concentrations of the ionic silver were prepared by serial dilution of the highest concentration.

At the end of treatment period, the cells were washed with HBSS again, swollen with 0.075 M KCl for approximately 15 s and fixed in cold methanol–acetic acid (25:1, v/v) and allowed to dry in the air. For evaluation of micronucleus formation, the cells were stained with 0.1% acridine orange for 3 min, rinsed briefly with phosphate-buffered saline and evaluated using an Olympus, model-BX43, fluorescent microscope (Olympus America, Center Valley, PA, USA). The slides were evaluated based on the OECD Guidelines for Testing Chemicals: In Vitro Mammalian Cell Micronucleus Test (OECD, 2014).

Statistical analysis

The statistical analysis of the cytotoxicity data was performed by John Ihrie, Biostatistics and Bioinformatics Staff, Office of Analytics and Outreach, Center for Food Safety and Applied Nutrition, FDA. A scatterplot was made for each endpoint/cell type combination using the open-source software R (http://www.r-project.org). Separate fixed-effects ANOVA tests were performed for each endpoint/cell type combination (positive controls were included). ANOVA tests were performed using the GLM procedure in SAS version 9.3 (Cary, NC, USA). A natural logarithm or arcsine transformation was used when needed to meet the ANOVA assumptions. Dunnett's test was used to adjust for multiple comparisons within each ANOVA analysis. For CBMN assay, Fisher's exact test ($P \leq 0.05$), performed by Dr.

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Shambhu Roy, was used for a pairwise comparison of the percentage of micronucleated cells in each treatment group with that of the vehicle control. The Cochran–Armitage trend test, performed by Dr. Shambhu Roy, was used to assess dose-responsiveness.

Results

This study was undertaken to evaluate two *in vitro* models, human liver HepG2 and colon Caco2 cells, which have been widely used for decades in the toxicity testing of conventional chemical toxicants, for predictive rapid genotoxicity screening tools with three objectives: (i) to evaluate the genotoxic potential of 50 nm nanosilver; (ii) to access the contribution of ionic silver to the genotoxic potential of nanosilver; and (iii) to compare the results of the present study with that of our recently published study on 20 nm silver (Sahu *et al*., 2014b). Both the 20 nm nanosilver and the 50 nm nanosilver particles had the same shape, composition, surface properties and were obtained from the same commercial source. The same experimental conditions, cell types (HepG2 and Caco2) and genotoxic endpoint (CBMN) were used for all the studies.

Characterization of nanosilver

The 50 nm silver nanoparticles in citrate used in this study were characterized by DLS, TEM and ICP–MS analysis as described previously for the 20 nm nanosilver (Sahu *et al*., 2014a, 2014b). The TEM images of 50 nm nanosilver showed no noticeable aggregation or agglomeration (Sahu *et al*., 2015). The silver nanoparticle used in our study was stable in the cell culture medium, which is in agreement with the stability reports of the 20 nm nanosilver (Sahu *et al*., 2014a, b) as well as the results of another independent laboratory evaluating the citrate-coated silver nanoparticles (Powers *et al*., 2011). The average size of the 50 nm nanosilver determined by TEM and DLS was 44.7 and 54.9 nm, respectively (Sahu *et al*., 2015), close to the manufacturer-provided value of 50.0 nm. We determined the concentration of silver in the 50 nm nanosilver solution used in this study by the ICP–MS analysis. The silver concentration of 0.984 mg ml^{-1} determined by us (Sahu *et al*., 2015) was similar to the manufacturer-provided concentration of 1.00 mg ml^{-1}. The dosing solutions in our studies were prepared based on the nanosilver concentration determined by our analysis.

Cytotoxicity of nanosilver

The 50 nm nanosilver is significantly cytotoxic to both the HepG2 and Caco2 cells in the concentration range of 10.0 to 50.0 μg ml^{-1} for 4-h exposures compared with the negative control (Table 1). For 24-h exposure, this nanosilver is significantly cytotoxic to HepG2 cells in the concentration range 5–50 μg ml^{-1} but at a higher concentration range 10 to 50 μg ml^{-1} for Caco2 (Table 1). It may be noted that the HepG2 cells were more susceptible to the 50 nm nanosilver exposure than the Caco2 cells (Table 1) like the smaller 20 nm nanosilver (Sahu *et al*., 2014a). The ionic silver (silver acetate) is significantly cytotoxic to both HepG2 and Caco2 cells in the concentration range of 0.5 to 3.0 μg ml^{-1} for 4-h exposure (Table 2) but at a lower concentration range of 0.2 to 3.0 μg ml^{-1} for the 24-h exposure (Table 2). It may be noted that the ionic silver is much more cytotoxic to both the cell types than the nanosilvers.

Genotoxicity of nanosilver

The genotoxicity of 50 nm nanosilver was evaluated by the CBMN assay with acridine orange staining and fluorescence microscopy as reported previously for the 20-nm silver nanoparticle (OECD, 2014; Sahu *et al*., 2014b). The OECD guidelines recommend that the highest test concentration for the CBMN assay should produce 55 ± 5% cytotoxicity (OECD, 2014). In our first attempt for evaluation of the genotoxic potential of 50 nm silver in HepG2 and Caco2 cells by the CBMN assay according to the OECD guidelines, we selected the highest concentration of the nanosilver based on the results of our cytotoxicity assays (Table 1). As close to 50% cytotoxicity was observed in HepG2 cells exposed to 50 μg ml^{-1} of the 50 nm nanosilver (Table 1), we selected 20 μg ml^{-1} as its highest test concentration for the 4-h exposure and 5 μg ml^{-1} for the 24-h exposure for our first experiment. Similarly, for the Caco2 cells, we selected 20 μg ml^{-1} as the top test concentration of the 50 nm nanosilver both for 4 and 24-h exposures for our first experiment. Our first CBMN experiment, which followed the OECD guidelines, did not result in significant induction of MN in either cell types. Therefore, for our subsequent CBMN experiments, we followed the recommendations of Elespuru *et al*. (2009) and ICH (International Conference on Harmonization, 2008); ICH (International Conference on Harmonization), 2011) that allow the use of test concentrations higher than recommended by the OECD guidelines for genotoxicity testing of potential genotoxicants to assure their hazard identification required for safety assessment. In our subsequent experiments, we repeated the CBMN assay in HepG2 and Caco2 cells using higher concentrations of the 50 nm nanosilver with the hope that we may find MN formation induced by the 50 nm silver at high test concentrations. The results of our subsequent CBMN assays in HepG2 and Caco2 cells treated with higher concentrations of 50 nm nanosilver are presented in

Table 1. Effect of 50 nm nanosilver on the viability of HepG2 and Caco2 cells

50 nm nanosilver concentration (μg ml^{-1})	HepG2 cell viability (% of negative control)	Caco2 cell viability (% of negative control)
A. 4-h exposure		
0 (Negative control)	100.0 ± 12.8	100.0 ± 5.9
5	91.0 ± 6.4	96.0 ± 8.1
10	87.1 ± 6.5*	91.8 ± 6.3*
20	72.4 ± 7.5*	81.9 ± 5.1*
50	57.3 ± 5.7*	67.7 ± 6.8*
Etoposide, 10.0 μg ml^{-1} (Positive Control)	31.8 ± 3.1*	48.2 ± 3.7*
B. 24-h exposure		
0 (Negative control)	100.0 ± 5.5	100.0 ± 6.8
5	87.2 ± 7.4*	93.2 ± 6.5
10	82.7 ± 7.1*	82.6 ± 1.7*
20	68.4 ± 5.7*	75.2 ± 10.0*
50	53.2 ± 10.1*	58.4 ± 11.6*
Etoposide, 10.0 μg ml^{-1} (Positive Control)	24.3 ± 1.8*	37.1 ± 13.2*

The viability of cells, exposed to ionic silver, was determined by the resazurin assay. Each value is the mean ± SD from 10 replicates.
* Different from the negative control ($P \leq 0.05$).

Table 2. The effect of ionic silver on the viability of HepG2 and Caco2 cells

Ionic silver concentration ($\mu g\ ml^{-1}$)	HepG2 Cell viability (% of negative control)	Caco2 Cell viability (% of negative control)
A. 4-h exposure		
0 (Negative control)	100.00 ± 8.2	100 ± 6.6
0.2	91.7 ± 6.8	85.0 ± 11.6
0.5	82.0 ± 4.0*	76.9 ± 12.8*
1.0	74.2 ± 4.9*	67.2 ± 9.8*
2.0	62.3 ± 5.9*	50.3 ± 4.9*
3.0	51.8 ± 5.8*	36.6 ± 5.4*
Etoposide, 10.0 μg/ml (Positive Control)	34.0 ± 4.2*	48.6 ± 7.1*
B. 24-h exposure		
0.0 (Negative control)	100.0 ± 6.8	100.0 ± 10.5
0.2	83.9 ± 3.3*	80.4 ± 8.0*
0.5	73.9 ± 9.3*	72.1 ± 4.0*
1.0	67.6 ± 8.4*	64.1 ± 5.8*
2.0	54.8 ± 7.3*	44.6 ± 6.5*
3.0	42.7 ± 8.6*	29.0 ± 2.4*
Etoposide, 10.0 μg/ml (Positive Control)	25.1 ± 2.4*	38.4 ± 4.3*

The viability of cells, exposed to ionic silver, was determined by the resazurin assay.
Each value is the mean ± SD from 10 replicates.
* Different from the negative control ($P \leq 0.05$).

Figs. 1–4. Figures 1 and 2 show CBPI and MN formation in HepG2 cells exposed to the nanosilver for 4 and 24 h, respectively. Similarly, Figs. 3 and 4 show CBPI and MN formation in Caco2 cells exposed to the nanosilver for 4 and 24 h, respectively. In 4-h exposure of HepG2 cells, a flat response in the reduction of CBPI was observed ranging from 18% to 23% between the concentrations of 25 to 100 $\mu g\ ml^{-1}$ (Fig. 1). In 24-h treatment of HepG2 cells, a flat cytotoxic response was observed ranging from 19% to 33% between the concentrations of 2.5 to 25 $\mu g\ ml^{-1}$ (Fig. 2). Similarly, in 4-h exposure of Caco2 cells, a flat response in the reduction of CBPI was observed ranging from 29% to 39% between the concentrations of 10 to 75 $\mu g\ ml^{-1}$ (Fig. 3). At the highest concentration of 50 nm silver(100 $\mu g\ ml^{-1}$) used for 4-h exposure, the reduction in CBPI was ~80% and, therefore, we did not evaluate the MN formation in order to avoid the false-positive results (Hillard *et al.*, 1998). In the 24-h treatment of Caco2 cells, a flat cytotoxic response was observed ranging from 19% to 33% between the concentrations of 2.5 to 25 $\mu g\ ml^{-1}$ (Fig. 4). These observations indicate increasing the concentrations of nanosilver do not have much impact on the reduction of CBPI in these cell types. Our data suggest that 50 nm nanosilver does not induce micronucleus formation in either HepG2 or Caco2 cells in the higher concentration range (10–100 $\mu g\ ml^{-1}$ for 4 h and 2.5–25 $\mu g\ ml^{-1}$ for 24-h exposures) tested. This is the reason why we did not evaluate the MN formation at the highest test concentration (100 $\mu g\ ml^{-1}$) of 50 nm silver in HepG2 or Caco2 cells at 4- or 24-h exposure to avoid the false-positive results (Hillard *et al.*, 1998). We found no correlation between increasing 50 nm nanosilver concentration and cytotoxicity based on CBPI in either of the cell types up to 100 $\mu g\ ml^{-1}$ in the 4-h treatment (Fig. 5) and up to 25 $\mu g\ ml^{-1}$ in the 24-h treatment (Fig. 6).

The effects of the ionic silver (silver acetate) exposure on HepG2 cells are presented in Table 3. At 4-h exposure of the ionic silver, the MN containing HepG2 cell population increased significantly by 1.8-fold at the top concentration of 2.5 $\mu g\ ml^{-1}$ compared with the negative control. At concentrations higher than 2.5 $\mu g\ ml^{-1}$, the cells died as a result of excessive cytotoxicity. Similarly at 24 h exposure of ionic silver, the MN-containing HepG2 cell population increased significantly by 1.9-fold at the top concentration of 2.0 $\mu g\ ml^{-1}$ compared with the negative control. At concentrations higher than 2.0 $\mu g\ ml^{-1}$ the cells died owing to excessive cytotoxicity. It may be noted that 2.0 $\mu g\ ml^{-1}$ represents 55–62% HepG2 cell viability (Table 2) very close to the highest test concentration (55 ± 5% cytotoxicity) recommended by the OECD guidelines 487. Besides, the concurrent cytotoxicity assessment using the CBPI method in the CBMN assay also showed similar excessive cytotoxicity at the concentration where the MN induction was significantly higher.

The effects of the ionic silver (silver acetate) exposure on Caco2 cells are presented in Table 4. At 4-h exposure of ionic silver, the MN containing Caco2 cell population increased significantly by

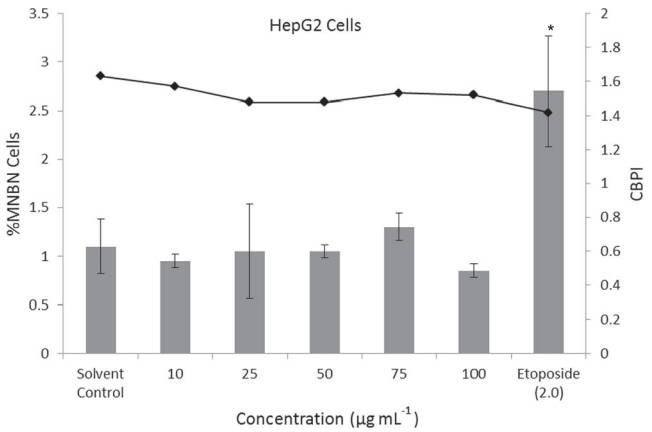

Figure 1. CBPI and % MN in BN cells of HepG2 cells treated with 50 nm silver for 4 h. The graph shows the values of the frequency of MNBN cells (grey bar) and the proliferation marker CBPI (black line). *$P < 0.05$ versus the solvent control (Fisher's Exact test). CBPI, cytokinesis-block proliferation index; MN, micronucleus; BN, binucleated cells.

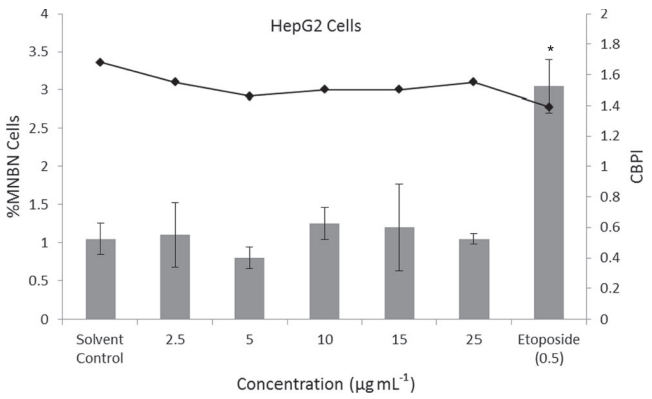

Figure 2. CBPI and %MN in BN cells of HepG2 cells treated with 50 nm silver for 24 h. The graph shows the values of the frequency of MNBN cells (grey bar) and the proliferation marker CBPI (black line). *$P < 0.05$ versus the solvent control (Fisher's Exact test). CBPI, cytokinesis-block proliferation index; MN, micronucleus; BN, binucleated cells.

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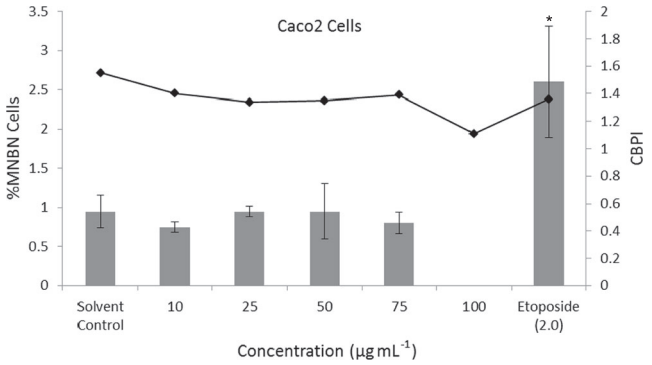

Figure 3. CBPI and %MN in BN cells of Caco2 cells treated with 50 nm silver for 4 h. The graph shows the values of the frequency of MNBN cells (grey bar) and the proliferation marker CBPI (black line). MN was not evaluated at the 100 μg ml^{-1} concentration because of an approximately 80% reduction in the CBPI indicating excessive cytotoxicity. *$P < 0.05$ versus the solvent control (Fisher's Exact test). CBPI, cytokinesis-block proliferation index; MN, micronucleus; BN, binucleated cells.

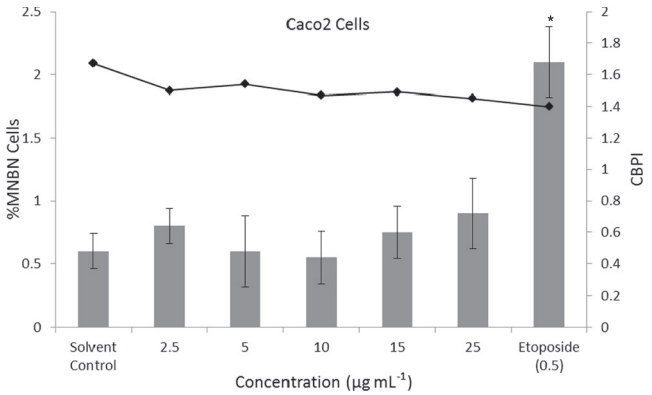

Figure 4. CBPI and %MN in BN cells of Caco2 cells treated with 50 nm silver for 24 h. The graph shows the values of the frequency of MNBN cells (grey bar) and the proliferation marker CBPI (black line). *$P < 0.05$ versus the solvent control (Fisher's Exact test). CBPI, cytokinesis-block proliferation index; MN, micronucleus; BN, binucleated cells.

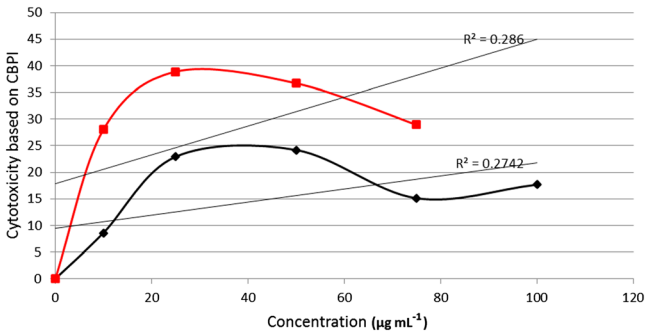

Figure 5. The cytotoxicity based on the reduction in CBPI for the 4-h treatment in HepG2 and Caco2 cells. The graph shows no correlation between cytotoxicity and increased concentration up to 100 μg ml^{-1} nanosilver. Linear regression was performed using Excel 2007. The black line ($R^2 = 0.2742$) = HepG2 cells. The red line ($R^2 = 0.286$) = Caco2 cells.

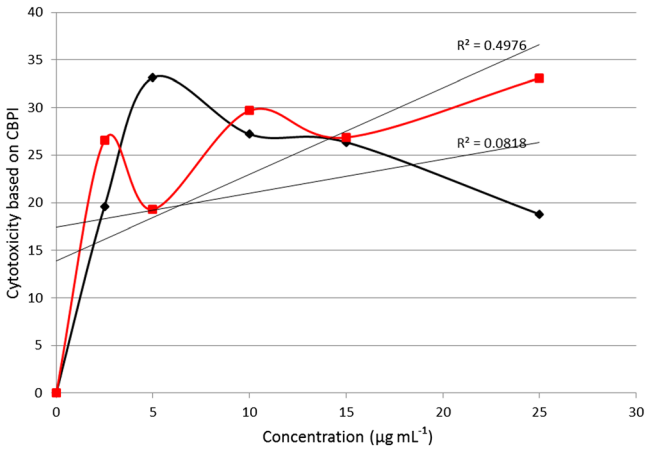

Figure 6. Cytotoxicity based on the reduction in CBPI for the 24-h treatment in HepG2 and Caco2 Cells. The graph shows no correlation between cytotoxicity and increased concentration up to 25 μg ml^{-1} nanosilver. Linear regression was performed using Excel 2007. The black line ($R^2 = 0.0818$) = HepG2 cells. The red line ($R^2 = 0.4976$) = Caco2 cells.

1.9-fold at the concentration of 0.5 μg ml^{-1} compared with the negative control. At concentrations higher than 0.5 μg ml^{-1} the cells died as a result of excessive cytotoxicity. Similarly at 24 h exposure of ionic silver, the MN-containing Caco2 cell population increased significantly by 2.8-fold at the concentration 2.0 μg ml^{-1} compared to the negative control. At concentrations higher than 2.0 μg ml^{-1} the cells died owing to excessive cytotoxicity. It may be noted that 2.0 μg ml^{-1} represents 45–50% Caco2 cell viability (Table 2). This concentration is close to the top test concentration (55 ± 5% cytotoxicity) of the ionic silver recommended by the OECD guidelines. Besides, the concurrent cytotoxicity assessment using the CBPI method in the CBMN assay also showed similar high cytotoxicity, at the concentration where the MN induction was significantly higher. It may be noted that the Caco2 cells are more susceptible to ionic silver compared with the HepG2 cells.

Discussion

The oral route of human exposure to nanosilver and its subsequent systemic absorption have been reported (Kim *et al.*, 2008). Recent *in vivo* studies show that the liver is a target organ for silver nanoparticles leading to hepatotoxicity (Sung *et al.*, 2009; Dziendzikowska *et al.*, 2012; Xue *et al.*, 2012). The HepG2 and Caco2 cells, representing the liver and gastrointestinal tract, respectively, have been widely used for decades as *in vitro* models for toxicity testing of classic toxicants (Brandon *et al.*, 2006; O'Brien *et al.*, 2006; Xu *et al.*, 2008; Jennen *et al.*, 2010). The *in vitro* CBMN assay has been used as a sensitive assay for genotoxicity testing of chemical and pharmaceutical toxicants (Fenech, 2000, 2007; OECD, 2014). Therefore, we are making an attempt to evaluate these two cell types in culture and the CBMN assay as rapid predictive screening tools for the evaluation of the genotoxic potential of food- and chemical-related nanosilver (Sahu *et al.*, 2014b). To our knowledge, our studies are the first attempt to characterize these two cell types to assess the potential genotoxicity of food- and cosmetics-related nanomaterials evaluated by the CBMN assay. Using these two cell types and the CBMN genotoxic assay, we have recently demonstrated that they are useful tools for rapid screening of nanosilver genotoxicity (Sahu *et al.*, 2014b).

We characterized the 50 nm nanosilver using the same methods as the 20 nm nanosilver reported previously (Sahu *et al.*, 2014a, b).

Table 3. The genotoxic effect of ionic silver on HepG2 cells

Ionic silver concentration ($\mu g\ ml^{-1}$)	CBPI	BN Cells containing MN (%)	MN Fold increase
A. 4-h exposure			
0.0	1.62 ± 0.02	1.10 ± 0.14	1.0
0.1	1.61 ± 0.01	1.25 ± 0.21	1.1
0.2	1.53 ± 0.01	1.4 ± 0.14	1.3
0.5	1.51 ± 0.02	1.3 ± 0.28	1.2
1.0	1.46 ± 0.03	1.65 ± 0.21	1.5
2.5	1.33 ± 0.01	2.0 ± 0.14	1.8*
5.0	Cells died		
Positive Control (Etoposide, 2.0 $\mu g\ ml^{-1}$)	1.45 ± 0.04	2.5 ± 0.28	2.3*
B. 24-h exposure			
0.0	1.6 ± 0.02	0.95 ± 0.21	1.0
0.5	1.44 ± 0.04	0.8 ± 0.14	0.8
1.0	1.45 ± 0.04	1.3 ± 0.28	1.4
2.0	1.32 ± 0.05	1.8 ± 0.14	1.9*
4.0	Cells died		
5.0	Cells died		
Positive Control (Etoposide, 0.5 $\mu g\ ml^{-1}$)	1.44 ± 0.05	2.1 ± 0.28	2.2*

The genotoxicity was evaluated by the CBMN assay counting 1000 BN cells containing MN. Each value is the mean ± SD of two replicates of a representative slide.

* Significantly different from the negative control ($P \leq 0.01$; one-sided Fisher's Exact Test).

CBMN, cytokinesis-block micronucleus; BN, binucleated; MN, micronucleus; CBPI, cytokinesis-block proliferation index.

Table 4. The genotoxic effect of ionic silver on Caco2 cells

Ionic silver concentration ($\mu g\ ml^{-1}$)	CBPI	BN Cells containing MN (%)	MN Fold increase
A. 4-h exposure			
0.0	1.49 ± 0.03	1.05 ± 0.21	1.0
0.1	1.46 ± 0.07	0.95 ± 0.21	0.9
0.2	1.44 ± 0.04	1.4 ± 0.42	1.3
0.5	1.29 ± 0.08	1.95 ± 0.21	1.9*
2.5	Cells died		
5.0	Cells died		
Positive Control (Etoposide, 2.0 $\mu g\ ml^{-1}$)	1.36 ± 0.06	2.4 ± 0.28	2.3*
B. 24-h exposure			
0.0	1.58 ± 0.02	0.75 ± 0.21	1.0
0.5	1.53 ± 0.02	1.1 ± 0.14	1.5
1.0	1.31 ± 0.07	1.3 ± 0.14	1.7
2.0	1.26 ± 0.02	2.1 ± 0.42	2.8*
4.0	Cells died		
5.0	Cells died		
Positive Control (Etoposide, 0.5 μg/ml)	1.24 ± 0.02	2.25 ± 0.21	3.0*

Genotoxicity was evaluated by the CBMN assay counting 1000 BN cells containing MN.

Each value is the mean ± SD of two replicates of a representative slide.

* Significantly different from the negative control ($P \leq 0.01$; one-sided Fisher's Exact Test).

CBMN, cytokinesis-block micronucleus; BN, binucleated; MN, micronucleus; CBPI, cytokinesis-block proliferation index.

The TEM images of 50 nm nanosilver presented in this report and those of 20 nm nanosilver reported previously (Sahu *et al.*, 2014a, b) showed no noticeable aggregation or agglomeration. Both the silver nanoparticles used in our studies were stable in the cell culture medium, which is in agreement with previous stability reports of citrate-coated silver nanoparticles (Powers *et al.*, 2011).

We evaluated the cytotoxicity of 50 nm nanosilver in HepG2 and Caco2 cells using the same methods as reported previously for 20 nm nanosilver (Sahu *et al.*, 2014a, b). A comparison of the cytotoxicity results of 50 nm nanosilver with those of the 20 nm nanosilver previously published (Sahu *et al.*, 2014a) shows that the smaller (20 nm) nanosilver particle is more cytotoxic than the larger (50 nm) one. Also, our studies show that the HepG2 cells are more susceptible to both the nanosilver particles than the Caco2 cells (Sahu *et al.*, 2014a). Vrcek *et al.* (2014) used nanosilver, prepared in house, and evaluated its cytotoxicity in HepG2 cells. They found the nanosilver particles taken up by the cells as we have reported earlier (Sahu *et al.*, 2014a, b). Their nanosilver particles were cytotoxic to HepG2 cells (Vrcek *et al.*, 2014) similar to our results reported earlier (Sahu *et al.*, 2014a).

We evaluated the genotoxicity of 50 nm nanosilver in HepG2 and Caco2 cells using the same experimental conditions and the genotoxic endpoint (CBMN) as reported previously for the 20 nm nanosilver (Sahu *et al.*, 2014b) according to the OECD guidelines (OECD, 2014). Our preliminary experiment was designed bearing in mind the reports of Karlsson *et al.* (2009) and Sahu *et al.* (2014a, b). Therefore, in our first preliminary genotoxicity experiment of 50 nm nanosilver we used the test concentration range and all other experimental conditions close to that of 20 nm nanosilver (Sahu *et al.*, 2014b). However, in this concentration range (0.0 to 20 $\mu g\ ml^{-1}$) it did not result in micronucleus formation in either of the cell types. It may be noted that under such circumstances the use of higher concentrations of potential genotoxicants is recommended in CBMN tests to assure their hazard identification required for safety assessment (Elespuru *et al.*, 2009; ICH 2008, 2011). Therefore, we repeated the CBMN genotoxicity assay for the 50 nm silver at a higher concentration range (10.0 to 100 $\mu g\ ml^{-1}$) bearing in mind that the high concentrations might result in false-positive results (Hillard *et al.*, 1998). Even higher test concentrations of 50 nm silver did not induce MN formation in HepG2 or Caco2 cells at 4- or 24-h exposures compared with the controls. The CBPI and MN formation in HepG2 and Caco2 cells induced by the 20 nm nanosilver under the same experimental conditions as the 50 nm nanosilver have been reported previously (Sahu *et al.*, 2014b). The 20 nm silver induced significant increases in MN formation in both the cell types compared with the control. The micronucleus formation in both HepG2 and Caco2 cells induced by 20 nm nanosilver was dependent on the exposure time and nanosilver concentration. The frequency of MN formation in HepG2 cells increased to 3.2% and 3.5% after 4- and 24-h exposure, respectively,

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from the control background frequency of 1.5%. Similarly, the frequency of MN formation in Caco2 cells increased to 2.7% and 3.4% after 4- and 24-h exposure, respectively, from the control background frequency of 1.4%. The results of our comparative genotoxicity studies on the 50 nm nanosilver (presented in this report) and those of the 20 nm silver reported previously (Sahu *et al*., 2014b) show that the smaller (20 nm) nanosilver is genotoxic to both HepG2 and Caco2 cells, but the larger (50 nm) one shows a much weaker response compared with the control under the same experimental conditions.

Recent studies indicate that the cellular response to nanoparticles may be affected by the presence and physicochemical properties of a protein adsorption layer (protein corona) formed around the nanoparticles (Gebauer *et al*., 2012; Treuel *et al*., 2014). The nanoparticle size and surface properties determine the formation of its protein corona (Cedervall *et al*., 2007; Lundqvist *et al*., 2008). Cedervall *et al*. (2007) evaluated the protein corona formation with a series of copolymer nanoparticles of various sizes using albumin as a model protein. They found that albumin formed coronas with 620 protein molecules per 70 nm particle compared with 4650 proteins per 200 nm particle (Cedervall *et al*., 2007). Lundqvist *et al*. (2008) investigated the effect of nanoparticle size and surface charge on the formation of the protein corona from human plasma using 50- and 100-nm polystyrene nanoparticles. They observed aggregation of the larger 100 nm particles compared with the smaller ones (Lundqvist *et al*., 2008). In our study, we used two nanosilver particles of different sizes (20 and 50 nm), but of the same shape, composition, surface charge and obtained from the same commercial source. Therefore, we hypothesize that the larger 50 nm nanosilver has an associated surrounding protein corona that makes the total 'size' of the particle either more susceptible to aggregation or reduces the larger particles ability to translocate into the cell, therefore, reducing toxicity. This could be similar to the protein corona effect noted by Cedervall *et al*. (2007) and Lundqvist *et al*. (2008).

Our results on the induction of micronuclei formation in HepG2 and Caco2 cells by 20-nm silver citrate nanoparticles agree with the results reported previously by AshaRani *et al*. (2009); Kawata *et al*. (2009) and Xu *et al*. (2012). AshaRani *et al*. (2009) who reported micronucleus formation in human lung fibroblasts and human glioblastoma cells exposed to the 6–20 nm starch-capped silver nanoparticles. Kawata *et al*. (2009) reported micronucleus formation induced by 7–10 nm silver nanoparticles in HepG2 cells. Xu *et al*. (2012) observed micronucleus formation in HeLa cells induced by 5–30 nm silver nanoparticle-hydrogel exposure. The results of our findings on 20 nm silver as well as the findings of other independent laboratories (AshaRani *et al*., 2009; Kawata *et al*., 2009; Xu *et al*., 2012) support the concerns about the safety of nanosilver found in food-related consumer products. Our results suggest that nanosilver, at least of sizes less than 30 nm shown in these published reports (AshaRani *et al*., 2009; Kawata *et al*., 2009; Xu *et al*., 2012; Sahu *et al*., 2014b, and 2015), has the potential for genotoxicity. Our studies show that both the HepG2 and Caco2 cells in culture represent two sensitive model *in vitro* systems for use in predictive screening for cytotoxic and genotoxic potential of food-related nanoparticles. They can serve as useful tools to study the mechanisms of nanoparticle-induced cytotoxicity and genotoxicity.

There is an ongoing debate on the contribution of ionic silver to the nanosilver toxicity (Lubick, 2008; Bae *et al*., 2010; Ho *et al*., 2010; Sotirious and Pratsinis, 2010; Powers *et al*., 2011; Jiao *et al*., 2014; Jiang *et al*., 2015). In our study reported here, we evaluated the genotoxic potential of ionic silver (silver acetate) to determine its contribution to the observed genotoxicity of nanosilver using the same *in vitro* model, the CBMN genotoxic endpoint and experimental conditions (Sahu *et al*., 2014b). For this study, we followed the OECD guidelines (OECD, 2014), which recommend that the top concentration of the test agent evaluated for micronucleus induction should produce approximately 55 ± 5% cytotoxicity, as well as the recommendations of Elespuru *et al*. (2009) and ICH (2008, 2011) for the use of higher concentrations of potential genotoxicants to assure the hazard identification required for its safety assessment. We evaluated the cytotoxicity of ionic silver (silver acetate) in HepG2 and Caco2 cells using the same methods as reported previously for the nanosilver (Sahu *et al*., 2014a). A comparison of the cytotoxicity results of ionic silver with those of the nanosilver previously published (Sahu *et al*., 2014a) shows that the ionic silver is more cytotoxic to both the cell types compared with the nanosilver. In the case of ionic silver, we found Caco2 cells to be more susceptible to the ionic silver than the HepG2 cells. This observation is exactly opposite to our observation with the nanosilver (Sahu *et al*., 2014a), where HepG2 cells were more susceptible than the Caco2 cells under the same conditions. These intrinsic properties of the HepG2 and Caco2 cells suggest that ionic silver is not responsible for the cytotoxicity of nanosilver. Our results agree with the reports from other independent laboratories that the ionic silver does not contribute to the toxicity of nanosilver (Sotirious and Pratsinis, 2010; Powers *et al*., 2011; Jiao *et al*., 2014). Vrcek *et al*. (2014) used silver nitrate as the source of ionic silver and came to the conclusions similar to ours. They found that ionic silver is much more cytotoxic to HepG2 cells than the nanosilver.

Micronucleus (MN) is formed if the entire chromosome or its broken pieces are not incorporated into the daughter nuclei during mitosis (Samanata and Dey, 2012). In this study, we evaluated the genotoxicity of ionic silver (silver acetate) by its ability to form MN in HepG2 and Caco2 cells using the same experiment conditions and the CBMN assay as reported previously for nanosilver (Sahu *et al*., 2014b). Currently, there is no international consensus on the analysis of the data. The OECD guidelines (OECD, 2014) indicate that the concentration-related increase or a statically significant increase in MN-containing cells can be used for determining a positive result. Bryce *et al*. (2008) have used an associated threefolds increase over the negative control to determine positive MN responses for conventional test chemicals using a flow cytometry-based method. Recio *et al*. (2012) have used ≥ twofolds increase over the negative control according to the OECD guidelines (OECD, 2014). We used their criteria to determine the MN response of ionic silver in HepG2 and Caco2 cells in this study.

In the cellular systems, genotoxicity and cytotoxicity are intimately correlated. It may be noted that excessive cytotoxicity could lead to false-positive genotoxicity in *in vitro* genotoxicity testing (Galloway, 2000; Kirkland *et al*., 2007; Bryce *et al*., 2010). Therefore, the primary concern for genotoxicity testing is the potential contribution of excessive cytotoxicity to produce false-positive results (Galloway, 2000; Kirkland *et al*., 2007; Shi *et al*., 2010; Bryce *et al*., 2010).

In our study, the ionic silver induces greater than twofolds increase in MN formation only in Caco2 cells after 24-h exposure at a concentration 2.0 $\mu g\ ml^{-1}$, which is close to the top concentration allowed by the OECD guidelines (OECD, 2014). The Caco2 cells showed a significant barely close to a twofolds increase in MN formation at 4-h exposure of ionic silver at a concentration (0.5 $\mu g\ ml^{-1}$) lower than the top test concentration recommended by the OECD guidelines. We believe that these are false-positive results produced by excessive cytotoxicity (Galloway, 2000;

Kirkland *et al.*, 2007; Bryce *et al.*, 2010). At excessive cytotoxic concentrations many of the normal cellular functions get impaired which leads to activation of necrosis and apoptotic pathways (Larrick and Wright, 1990). Many intracellular pathways are activated by the binding of cytokines to the cellular membrane that could lead to G protein-coupled activation of phospholipases, generation of free radicals and damage to the nuclear DNA by endonucleases. In the present study, in HepG2 cells at 4-h treatment, a statistically, significant induction was observed only at one concentration (2.5 μg ml–1) with 47% cytotoxicity whereas at a lower concentration (1 $\mu g\ ml^{-1}$), the response was negative. Moreover, at next higher concentration (5 $\mu g\ ml^{-1}$), ionic silver was completely cytotoxic. A very close pattern of cytotoxicity and the genotoxic response was observed in HepG2 and Caco2 cells at the 4- and 24-h treatment condition underlining that ionic silver (silver acetate) is not a potent genotoxicant and the observed response could be secondary to cytotoxicity. Therefore, we conclude that the ionic silver (silver acetate) does not behave like a classic genotoxicant as evaluated by the CBMN assay. Our results on the induction of MN formation in HepG2 and Caco2 cells by ionic silver appear to be similar to the results reported by Jiao *et al.* (2014); Powers *et al.* (2011) and Sotirious and Pratsinis (2010), which demonstrate that ionic silver does not contribute to the toxicity of nanosilver.

Genotoxicity is an important endpoint of the safety assessment of regulated products, but no single test is available to detect all types of genotoxicity. Therefore, a battery of tests is recommended by the international harmonized guidance (ICH, 2011) for hazard identification of genotoxic agents. The *in vitro* CBMN assay is a useful tool for predictive screening of potential genotoxicants. We believe that our observations of MN formation by the ionic silver evaluated by the CBMN assay under excessive cytotoxicity conditions may be false positives (Galloway, 2000; Kirkland *et al.*, 2007; Bryce *et al.*, 2010). Therefore, we conclude that ionic silver may not be genotoxic by the CBMN assay because of its failure to behave like a classic genotoxicant. However, we are aware of the reports that some genotoxins do not induce a biological response at lower doses because of efficient repair systems and cell-cycle management pathways (Thomas *et al.*, 2013; Fukushima *et al.*, 2015).

The results of our studies presented here show that ionic silver may not contribute to the genotoxicity of nanosilver. Our results agree with the reports of Vrcek *et al.* (2014) showing that ionic silver does not contribute to the cytotoxicity of nanosilver. It may be noted that in our studies we used silver acetate as the source of ionic silver but Vrcek *et al.* (2014) used silver nitrate. However, our observations are same. It appears that the source of ionic silver does not matter. The ionic silver whether it comes from silver acetate or silver nitrate does not contribute to the genotoxic potential of the nanosilver.

Huk *et al.* (2014) have reported that the toxic potential of nanosilver may not always be size dependent. In their study, they synthesized the nanosilver (50, 80 and 200 nm) in their own laboratory and used the gene mutation and comet assays as endpoints of genotoxicity. They evaluated the genotoxicity of these three nanosilver particles in human lung carcinoma epithelial A549 and Chinese hamster lung fibroblast V79-4 cells. They have reported that nanosilver of all three sizes is taken up by the two cell types they used. Their study shows that the largest size (200 nm) nanosilver is the most genotoxic.

Results of our study presented here agree with the conclusions of other independent studies (Karlsson *et al.*, 2009; Huk *et al.*, 2014). The studies of Karlsson *et al.* (2009) suggest that it may not be always possible to generalize the genotoxic potential of all sizes of nanoparticles with the same chemical composition and the same all other factors. Studies by Huk *et al.* (2014) came to a similar conclusion for nanosilver. Our study indicates that cell types and genotoxic endpoints may play important roles in the genotoxic potential of nanosilver.

Conclusions

The results of present study support the hypothesis that the nanoparticle size and cell types are critical determinants of its genotoxicity. We compared the genotoxic potential of two different sizes (20 and 50 nm) of silver nanoparticles of the same shape, composition, surface charge and obtained from the same commercial source. We used the same experimental conditions, the same *in vitro* models (human-derived cells HepG2 and Caco2) and the same genotoxic endpoint (CBMN). Results of our study show that up to the concentrations tested in these cell types, the smaller (20 nm) nanosilver induces micronucleus formation in both the cell types but the larger (50 nm) nanosilver and the ionic silver provide much weaker response compared with controls under the same conditions. The human HepG2 and Caco2 cells in culture appear to be sensitive *in vitro* model systems for rapid screening of cytotoxic and genotoxic potential of nanoparticles. They can serve as useful tools to the study mechanisms of nanoparticle-induced cytotoxicity and genotoxicity.

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Conflict of interest

The authors did not report any conflict of interest.

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