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A N I N T E R N A T I O N A L J O U R N A L

Evaluation of the Toxicity of Silver/Silica and Titanium Dioxide Particles in Mammalian Cells

Michele Pittol[1*], Daiane Tomacheski [1e2], Douglas Naue Simões[1e2], Vanda Ferreira Ribeiro[1e2], Ruth Marlene Campomanes Santana[2].

[1] Softer Brasil Compostos Termoplásticos Campo Bom, Rio Grande do Sul, Brazil [2] PPGE3M, Universidade Federal do Rio Grande do Sul Porto Alegre Brazil

ABSTRACT

The increasing concern over the spread of diseases has lead to a high consumption of antimicrobial additives in the medical and industrial fields. Since these particles can lixiviate from loaded materials, the contact between this additive and mammalian cells can occur during manufacture, use and disposal of the products. Silver on fumed silica (AgNP_SiO_2) and titanium dioxide (TiO_2) can be used as antimicrobial additives that are applied in polymeric formulation. While these additives can inhibit bacteria, fungus and virus proliferation; they may also be harmful to humans. Standard toxicological studies were undertaken using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide), CBPI (cytokinesis-block proliferation index) and micronucleus assay using different sets of additive concentrations. The nanosize of the samples evaluated was confirmed by transmission electronic microscopy. No significant micronucleus frequency increase or cell viability reduction were observed with the exposure of L-929 murine fibroblast cells to AgNP_SiO_2 and TiO_2 particles at any of the tested concentrations. The non toxic effect of the analyzed particles can be explained by considering its agglomeration tendency, composition, and crystalline form. Further investigations should be done to understand the interference of agglomeration and how it affects the toxicological study.

Key words: titanium dioxide, silver, silica, toxicology, mammalian cell

[*] Author for correspondence: mipittoll@gmail.com

INTRODUCTION

For a vast array of commercial and medical applications, nanoscale silver (AgNP) and titanium dioxide (TiO_2) particles are produced and used in different sizes, shapes, crystalline forms, morphology, surface coating and so forth[1,2]. AgNP is the most used nanoparticle in products manufactured for the marketplace [3,4]. In dermatologic applications (such as aerosols, suspensions and emulsions), TiO_2 nanoparticles are the most widely used[5,6]. The growing consumption of these particles is mainly due to their biocide properties[7]. Such widespread use and the production of novel engineered materials raises concerns that the release and exposure to nano and microparticles from loaded materials may pose a risk to human health[8] and to the environment[9].

Once the incorporation of nano and micro-sized particles has been widely applied within consumer products, the toxicological evaluation of the potential risks of its chemicals, mainly during the early stages of product development is of interest. Particle characteristics such as shape, chemical composition, capping agent, carrier composition, crystalline form, and surface energy may have an impact on the interaction with cells[10] and influence the toxic potential[2]. For nanoparticles, as size decreases; increases the ion conductivity on the surface[11]. Also, because of their small size, these particles may enter plant and animals cells and reach critical areas in organelles tissues and organs[12]. Not nanoscale TiO_2 is considered to be a non-toxic biocide[13,14] and its rutile crystal form is recommended as safe to use in cosmetic and pharmaceutical applications[15].

Reckoning with the above discussions, a range of studies has been performed in normal and cancerous cell trials to determine the role of different particles in toxicity[2,16,17,18]. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5 diphenyltetrazolium bromide) is a metabolic status assay that evaluates reductive capacity of cells[19]. In micronucleus assay the genetic damage is reported by the number of cells containing micronuclei[20]. These pre-screening measurements are useful to provide evidence of the adverse cytotoxicity effects that certain chemicals can cause to living organisms[21].

The aim of this study is to evaluate the citotoxicity and genotoxicity of silver nanoparticles adsorbed on fumed silica ($AgNP\_SiO_2$) and comercial titanium dioxide ($TiO_{2)}$ to mammalian cells.

MATERIALS AND METHODS

Particle characterization

The additives nanosilver adsorbed on fumed silica (AgNP_925-SiO, silicon dioxide 98.0-99.4% and silver 0.1-2.0%), supplied by TNS Nanotecnologia Ltda., referred to herein as "$AgNP\_SiO_2$" and commercial rutile dioxide titanium (TiPure R-103), supplied by DuPont referred to herein as "TiO_2" were used in this study.

The qualitative analysis of mineral composition was determined by X-ray diffraction in a Pan Analytical X'pert PRO and X'PertHigh Score software. Particle size distribution was determined by laser diffraction, and the equipment used was a CILAS 1180 particle size analyzer, with scanning ranging from 0.04 μm to 2500 μm. $AgNP\_SiO_2$ and TiO_2 were predispersed in deionized water using ultrasound for 60 s.

To perform morphological analysis by scanning electron microscopy (SEM) the samples were deposited in a carbon type stuck to stub and metalized with gold. For image acquisition was used a SEM of field emission (SEM-FEG) (Inspect F50, FEI) with 20 kV, spot 3 and working distance (WD) of 10 mm. Transmission electron microscopy (TEM) was performed at Tecnai, G2 T20 at a voltage of 200 kV. The samples were prepared by mounting a drop of the ethanol suspension containing the

particles on a 300 mesh copper grid carbon film. The average particle diameter and size distribution were calculated using Image J version 1.40g software.

L-929 murine cell line

L-929 murine fibroblast cell line were purchased from cell bank from Rio de Janeiro – Cell Bank, and were grown in the presence of the cell culture medium containing Dulbecco's Modified Eagle's Medium (DMEM- Sigma-Aldrich) (89%) supplemented with 1% penicillin/streptomycin (Cultilab) and 10% fetal calf serum (FCS - Cultilab). The cell was derived from normal subcutaneous areolar and adipose tissue of a 100 day old male mouse.
MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) and cytokinesis-block proliferation index (CBPI) assays were performed to identify cytotoxicity effects in L-929 murine fibroblast cells exposed to silver nanoparticle (AgNP_SiO_2) and titanium dioxide (TiO_2).
Considering the literature reports connecting the particle size to triggering detrimental effects on mammalian cells and the differences in toxicity values between AgNP and TiO_2[22,23], different sets of concentration were tested for AgNP_SiO_2 and TiO_2 in MTT assay.

MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay

The MTT cell proliferation assay was performed according to ISO 10993-5 to evaluate the cytotoxicity of AgNP_SiO_2 and TiO_2 particles. The incubation of AgNP_SiO_2 and TiO_2 were performed with *Mus musculus* murine fibroblasts cell line L-929. The particle suspensions were performed in seven concentrations (15, 30, 60, 120, 250, 500, 1000 ppm/plate) of AgNP_SiO_2 and of TiO_2 dispersed in DMEM (Sigma-Aldrich). For each particle and concentration, the assay was performed in nine replicates and repeated on two independent experiments. Cells were also treated with doxorubicin (Sigma-Aldrich) in nine different concentrations (5; 2.5; 1.25; 0.62; 0.31; 0.15; 0.07; 0.03 and 0.01 μg/mL as a positive control. Cell suspensions were inoculated onto 96-well cell culture plates at 1 x 10^5 cells/mL, incubated in an humidified atmosphere of 5% CO_2/95% air at 37 °C for 24 hours to allow for cell adhesion and sedimentation. At the end of period, test metal particles and positive control doxorubicin (Sigma-Aldrich) were incubated with the cells for 24 hours. After the end of the exposure period, a 50 μL MTT mixture (0.5 mg/mL) was added into each well. They were again incubated in a humidified atmosphere of 5% CO_2/95% air at 37°C for 3 hours. Then the MTT mixture was removed and the precipitated formazan was dissolved in isopropyl ethanol.
Absorbance of the formazan product from each well was measured by spectrophotometer (SPECTRA max PLUS 384 Microplate reader, Molecular Devices) at 570 nm. Three controls were set up for each experiment: (1) blank - quality check of assay (phosphate buffered saline - PBS), (2) negative control (cells + DMEM) consisting of 100% cell viability[24] and (3) positive control consisting of doxorubicin (Sigma-Aldrich).
In viable cells the yellow tetrazolium salt is reduced by mitochondrial enzymes (succinate dehydrogenase) to a blue water-insoluble formazan product[24]. Citotoxicity values were obtained from the absorbance average (570 nm) of the replicates in each treatment (concentration) deducted from PBS values and compared to negative control values. Cytotoxicity was categorized according to Dahl *et al.*[25], as follow:
- More than 90% cell viability: not cytotoxic
- 60-90% cell viability: slightly cytotoxic

- 30-59% cell viability: moderately cytotoxic
- Less than 30% cell viability: severely cytotoxic

Cytokinesis-block proliferation index (CBPI) and micronucleus assay

Genotoxicity of AgNP_SiO_2 and TiO_2 particles to murine fibroblasts cell was evaluated based on OECD 487 cytokinesis-block proliferation index (CBPI) assay. The CBPI test was performed with *Mus musculus* murine fibroblasts cell line L-929. Three distinctive concentrations of AgNP_SiO_2 and TiO_2 particles (15, 30, 60 ppm/plate) were tested in CBPI and micronucleus assay. For each particle and concentration, two independent experiments were conducted, each of which performed in triplicate. The dispersion procedure was the same as that used for the MTT assay. Two controls were set up for each experiment: (1) negative control consisting of DMEM medium; and (2) positive control consisting of methyl methanesulfonate (MMS). Incubation was performed with 0.7 x 10^5 cells/mL in a 5 mL suspension containing DMEM medium (Sigma-Aldrich) (89%), 1% penicillin/streptomycin (Cultilab) and 10% fetal calf serum (Cultilab) in a humidified atmosphere of 5% CO_2/95% air at 37°C for 24 hours.
At the end of the period, the test particles and the positive control MMS (4 x 10^{-5} M) were incubated for 24 and 6 hours respectively. After the end of exposure period, the cells were incubated with cytochalasin B (Sigma-Aldrich) (3 µg/mL) for 42 hours. Then the cells were treated with a hypotonic solution of KCl 0.075M (37°C) for 15 minutes and fixed in ethanol:acetic acid solution (3:1) at 4 °C. The slides were covered with the acridine orange staining reagent (50µg/mL) and examined with fluorescence microscopy (Nikon Eclipse Ni H600L) with a blue light excitation filter (488 nm) and yellow light emission/barrier (575 nm) using oil immersion objective.
According to Fenech[26], only binucleated cells (2000 cells/spot) with intact nuclei, approximately equal scales, and with the same pattern of staining were analyzed. For cytotoxicity evaluation the cytokinesis-block proliferation index (CBPI) was used, which indicates the number of cells with 1, 2, 3 or more nuclei in 500 viable cells, calculated in 2 independent assays per treatment.
Genotoxicity was expressed as the average ± standard error of the percent of micronucleus events per 2000 cells; calculated in two independent assays per treatment.

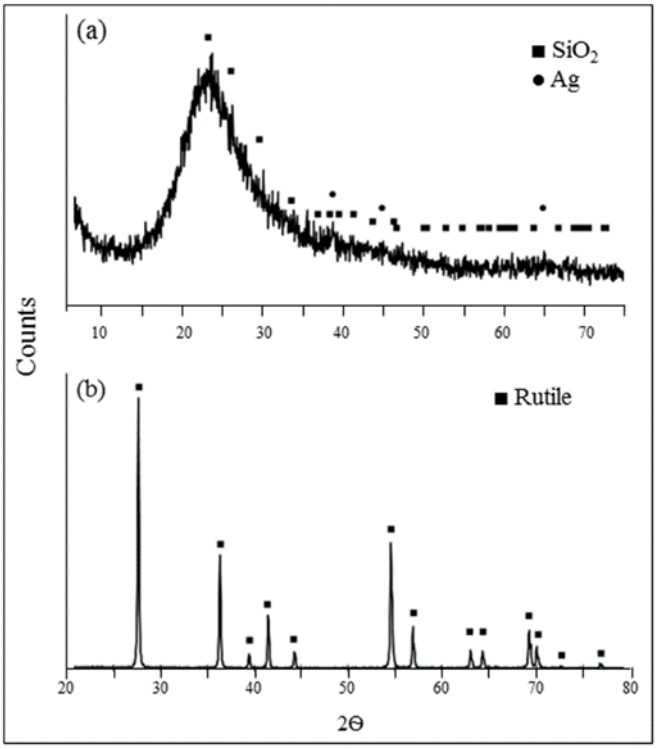
Statistics analysis

The results were expressed by average ± standard error of the mean and significance was obtained through statistical analysis of variance (ANOVA) following the Tukey's test if applicable. For all groups, $p < 0.05$ was considered as being statistically significant.

RESULTS

Particle characterization

The results of the X-ray diffraction detected the presence of SiO_2 and Ag in the AgNP_SiO_2 sample (Figure 1A). In accordance with diffractogram results, the SiO_2 in this sample was considered amorphous due to no diffraction peaks and a spreading halo with intensity of about $2\Theta = 22$ ° (characteristic of amorphous materials)[27,28]. The TiO_2 sample was confirmed as pure rutile (Figure 1B).



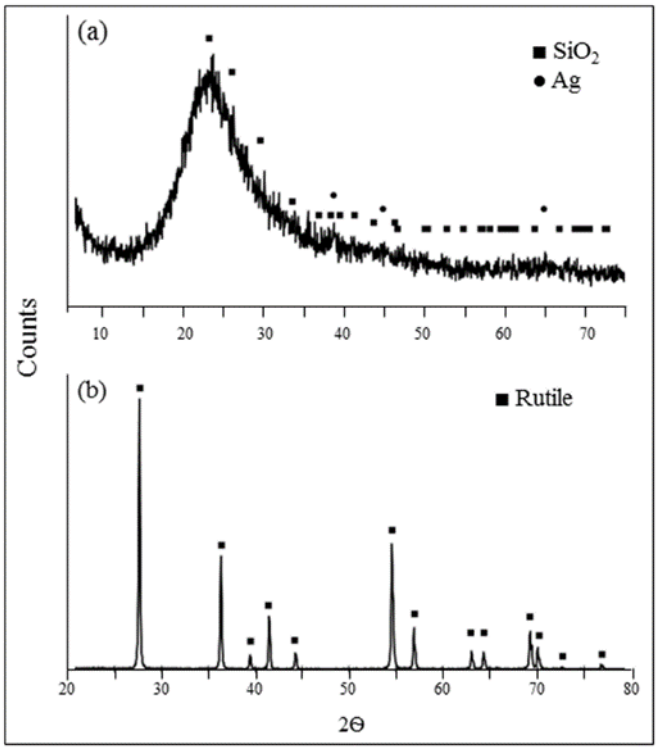


Figure 1- Diffractograms of AgNP_SiO_2 (A) and TiO_2 (B) samples.

Values of average diameters D_{10}, D_{50} and D_{90} determined by granulometric analysis are shown in Table 1.

Table 1- Values of average diameters D_{10}, D_{50} and D_{90} determined by laser diffraction.

	Average diameter, µm	D_{10}, µm	D_{50}, µm	D_{90}, µm
AgNP_SiO_2	12.97	4.7	9.2	28.99
TiO_2	0.29	0.08	0.25	0.54

It was noticed that the average size of the AgNP_SiO_2 (12.97µm) showed a value above the nanoscale (Table 1). This result reflects the size of the aggregates, as observed in SEM and TEM images (Figure 2A and Figure 2B). On SEM images, AgNP_SiO_2 was observed in the form of blocks with irregular geometry and size, with the same size determined by laser diffraction (between 5 and 30 µm). However, by observing TEM images it was possible to confirm nanoscale of this additive, with nanoparticles of silica (20 nm) and silver (10 nm), both in spherical forms (Figure 2B). The TiO_2 particles have an average size of 0.29 µm, but as seen in SEM images (Figure 2C), more particles have nanoscale, and the size determined by laser diffraction reflects the size of agglomerates. As shown in TEM images in Figure 2D, TiO_2 is of spherical form and average size of 90 nm.

The results found in laser diffraction and micrograph demonstrated that both additives have nanoscale and high tendency to agglomeration.

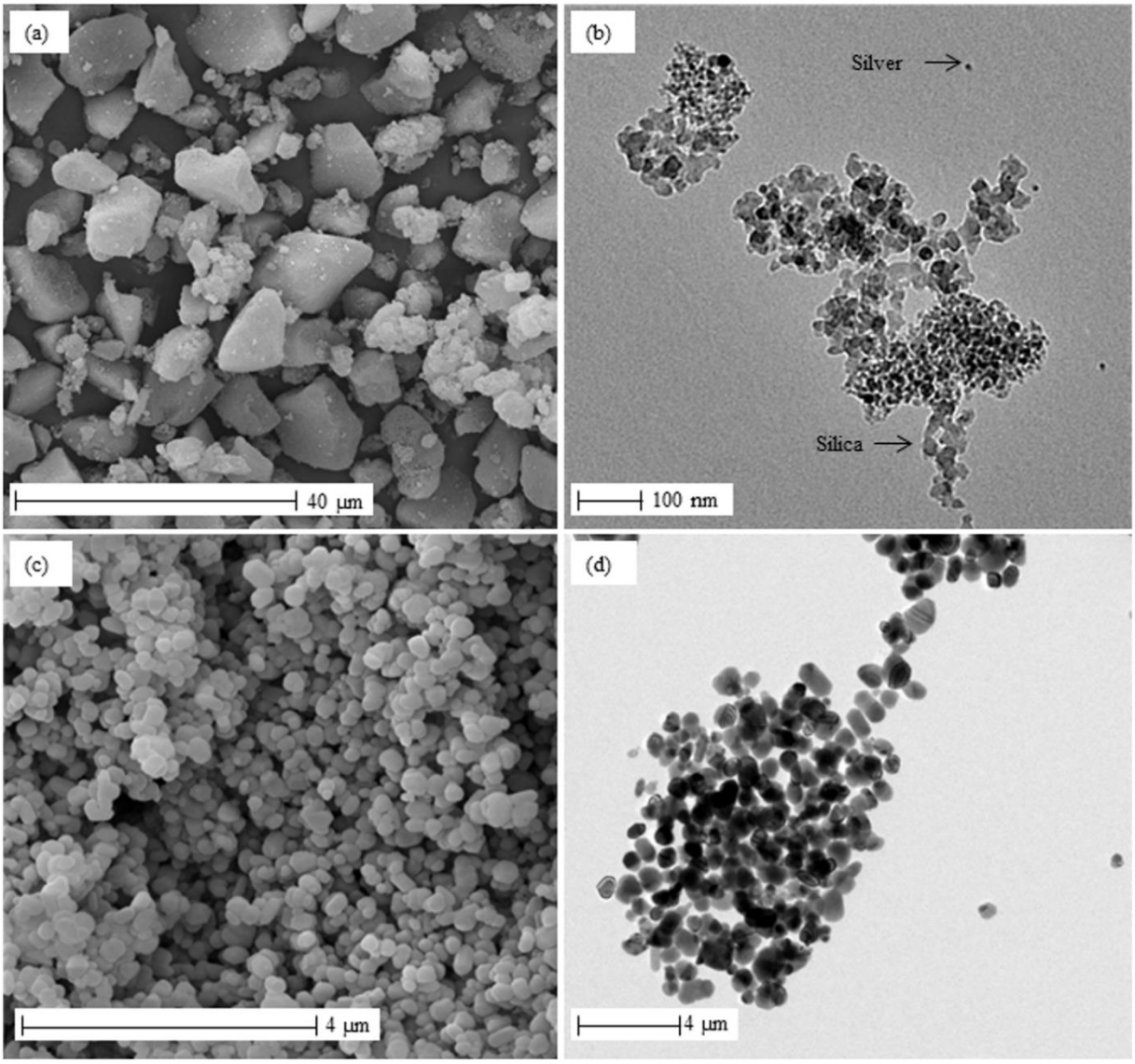


Figure 2- Micrographs of the SEM (left) and TEM (right) additives for (A, B) AgNP_SiO_2 and (C, D) TiO_2.

MTT data

Viability assay expound the cellular reaction to a toxicant[12]. The relation between test chemical (AgNP_SiO_2 and TiO_2), concentration (ppm) and cell viability (%) after 24 hour exposures using the MTT assay are presented in Figure 3.

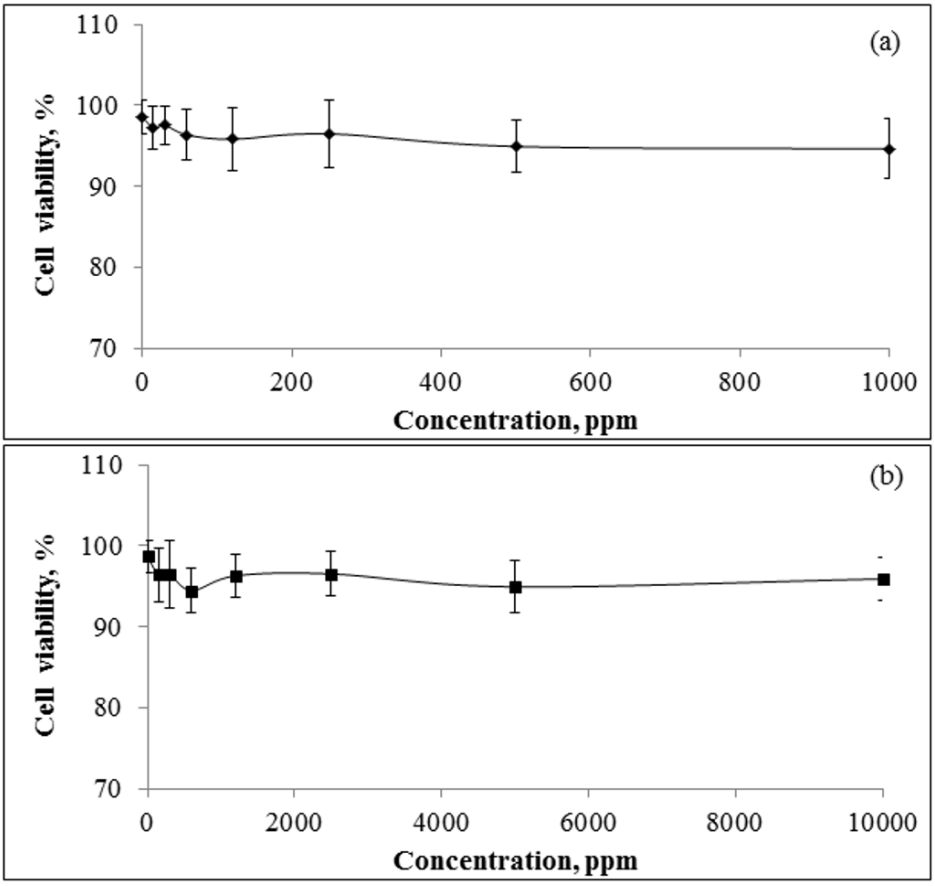


Figure 3- Cell viability of L-929 murine fibroblast after 24 h of exposure to distinctive concentrations of (A) AgNP_SiO_2 and (B) TiO_2 by MTT method. Each point represents the mean and standard deviation in nine replicates.

The viability of the cells exposed to AgNP_SiO_2 was significantly different ($p<0.05$) between the tested additive concentrations. In cell exposed to TiO_2 particles there was no difference between the concentrations of the additives. Since cell viability values were between 95.83 - 97.53 in the presence of AgNP_SiO_2 (Figure 3A) and 94.47 - 96.54 in cells treated with TiO_2 (Figure 3B), according to cell viability classification and the experimental conditions performed in this study, the AgNP_SiO_2 and TiO_2 particles were classified as non cytotoxic.

CBPI and micronucleus data

The experimental data for CBPI values and micronucleus frequency are summarized in Table 2. With data presented as mean values ± standard deviations. The experiment was conducted following 24 h exposure time.

No significant cell viability reduction was observed on murine cells treated with AgNP_SiO_2 and TiO_2. There were no concentration-dependent increases in micronucleus frequency on murine cells treated with AgNP_SiO_2 and TiO_2 (Table 2).

Table 2- CBPI values and micronucleus frequency of L-929 murine fibroblasts cells treated with $AgNP\_SiO_2$ and TiO_2 samples.

Treatment	Assay	
	CBPI[c]	
	$AgNP\_SiO_2$	TiO_2
NC[a]	1.67±0.016	1.67±0.016
PC[b]	1.11±0.023	1.11±0.023
15 ppm	1.66±0.020	1.65±0.009
30 ppm	1.64±0.010	1.66±0.008
60 ppm	1.65±0.008	1.68±0.008
	MN[d]	
	$AgNP\_SiO_2$	TiO_2
NC[a]	0.15±0.050	0.15±0.050
PC[b]	3.35±0.124	3.35±0.124
15 ppm	0.17±0.042	0.17±0.042
30 ppm	0.20±0.035	0.20±0.035
60 ppm	0.10±0.033	0.10±0.033

[a] negative control (DMEM), [b] positive control (MMS, $4x10^{-5}$ M), [c] cytokinesis-block proliferation index (CBPI) 500 cells analyzed, [d] micronucleus (MN) 2000 cells analyzed

DISCUSSION

The viability of L929 murine cell was found to be greater than 90%, even in the highest concentrations used in $NpAg\_SiO_2$ (average size - 10-20 nm; 1000 ppm) and TiO_2 (average size - 90 nm;10000 ppm) tests. In contrast to our findings, cytotoxic and genotoxic studies have shown harmful effects of nanosized (~0.1 to 100 nm) Ag and TiO_2 particles to mammals' cells[12]. A variety of scales and concentrations have been assigned to the AgNP[22,29,30] and TiO_2 toxicity values[23,31,32]. In a cytokinesis blocked micronucleus assay, DNA damage and significant numbers of micronuclei were observed in human fibroblast and human cancer cells after the treatment with AgNP (6-20 nm)[12]. In a research conducted by Li et al.[33] the AgNP (5 nm) particle induced significant increase in micronucleus frequency over the control. Carlson et al.[34] observed in 24 h MTT study a decrease of 88.66% in mitochondrial function of alveolar macrophages exposed to AgNP (15 nm) at concentrations of 50 µg/mL. However, bigger AgNP (55 nm) exhibit a viability decrease of 33.89% at the highest dose (75 ppm) and AgNPs (5-40 nm) in a concentration up to 100 ppm can be used as a drug delivery[18]. These above reports showed a variable degree of AgNP and TiO_2 toxicity, highlighting the complexity of the study of nanomaterials, emphasized by the diversity of the evaluated cells.

Concerns associated to nanoparticles are the metal ions released from the material[9], as well as their larger surface area, that enhance the interaction with cells[35], and the small size, that allows for easier penetration into the cells[36,37]. Although the engineering of NPs internalization into cells is considered as non-specific according to studies with microcopy images[38], endocytosis is an important mechanism to Ag[39] and TiO_2 nanoparticles[40] cell intake. However, particle agglomeration can mitigate the toxic effects of particle size, which may vary depending on cell type and internalization mechanisms[41,42]. In this study, micrographs and granulometric analysis show that AgNP_SiO_2 and TiO_2 present a tendency to agglomerate, with a set size of 12.97 µm and 0.29 µm, respectively. It is recognized that at the cellular level, size will have an impact on particle uptake[43]. In this way, as reported by Sambale et al.[22] non nanomaterial (above 0.1 µm) may prevent the cell apoptosis by diminishing access and penetration of particles into the cytoplasm, also the microparticles do not dissolve as rapidly as nanoscale ones, providing a slow release of metal, particularly around the nucleus of the cell[44].

The AgNP_SiO_2 presents the silica covering a range of 98.0-99.4% of the total particles (data provided by the suppliers). According to X-Ray diffraction, this silica is amorphous. Silica on amorphous structure is considered non toxic[45,46], also when silver nanoparticles are deposited on a silica carrier, the hydrophilicity characteristics of silica reduces the nonspecific binding of proteins[47] and the release of AgNP[48] leading to lower toxicity[49]. In a previous study, cell culture condition was shown to influence the toxicity of amorphous silica. Drescher and coworkers[50] observed an agglomeration tendency of silica nanoparticles in all FCS-containing medium, the same supplement used in this study, and this condition caused a decrease in toxicity. Such behavior may be explained by the organic components of treatment media[51], considering that the adsorption of serum proteins into silica surface can change particle compatibility, membrane contact and uptake into the cells[52]. Although micronucleus (MN) test has been considered more appropriate for evaluating the genotoxicity of the AgNP[33], there is an issue pertaining the use of cytochalasin B in MN assay. The addition of this chemical could mask the real cytotoxicity of a tested substance[44,53,54], by restricting particle uptake due the phagocytosis inhibition[55]. The alternative assays to access genotoxicity of nano and microparticles would be the Ames test and Comet assay[56]. However, in the case of this study, that evaluated the toxicity of known antimicrobial particles, the Ames test may not be a suitable option because its test uses bacterial cells for determining mutagenicity[57].

The uptake of small clusters by cells is more efficient than the uptake of larger clusters[58] and the presence of FCS in the MTT assay and cytochalasin B in MN test was shown to influence the formation of aggregates and therefore, may have an impact on the dispersion and bioavailability of the particles [59]. Thus, the non toxic effect of AgNP_SiO_2 and TiO_2 observed in our experimental conditions could be related to the agglomeration tendency of the tested particles[59,60], amplified by the contribution of treatment conditions used in the assay on the agglomeration and cell input mechanisms. Moreover, the TiO_2 rutile morphology is described as a less photocatalyst[61] and non-cytotoxic[15,62] form of TiO_2 crystal[63].

The murine fibroblast cell line L-929 used in this study is a suitable cell type for the investigation of *in vitro* toxicity of the metal particles, being routinely used for this purpose due its biological response, reproducible growth rates and facility in the cell culture conditions[64,65]. However, with respect to the sensitivity, this cell line were found to be resistant to plant extracts[66], but also sensitive to other substances[64,67]. In summary, it seems that apart of cell line, the elements that control nanoparticles cytotoxic responses vary depending on the concentration, size and type of material tested as well as the assay system. Mahmoudi and coworkers (2009)[68] reported enhancement of mitosis and apoptosis phenomena in G_0/G_1 phase after the exposure of

fibroblast cell to iron oxide nanoparticles. Also, cellular damage and DNA deterioration was reported upon the exposure to TiO_2[40] and Ag nanoparticles[69], and the concentration of these nanoparticles was reported inside mitochondria, nucleus and nucleolus[12].

At this point, it can be speculated that the manner in which the particle is presented to the cell in terms of supporting elements (e.g. SiO_2), crystal form, particle and agglomerate sizes, as well as the treatment medium conditions could be the factors handling this toxicological study. Additionally, the comparison of our results with others was hampered due to miscellaneous methods, concentrations, cell types and mainly the characteristics of the materials used in previous toxicological inventories. Due to the diversity of structural components, the committee on emerging and newly identified health risks - SCENIHR[70] advocates that the toxicological assessment of novel materials (such as AgNP_SiO_2) should be done on a case-by-case basis.

CONCLUSION

The particles AgNP_SiO_2 and TiO_2 did not cause citotoxicity and genotoxicity detectable by MTT and CBPI tests in L-929 murine fibroblast cell at any tested concentration. The non toxic effect of the analyzed particles can be explained by considering its agglomeration tendency, supporting element, and crystalline form, together with their interaction in culture media. Clearly, further investigations are needed to better understand the cyto/genotoxicity mechanisms of AgNP_SiO_2 and TiO_2 in cultured mammalian cells.

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