International Journal of Research in Medical Sciences
Ganapathi AP et al. Int J Res Med Sci. 2014 Nov;2(4):1360-1367
www.msjonline.org
pISSN 2320-6071 | eISSN 2320-6012
DOI: 10.5455/2320-6012.ijrms20141122

Research Article

In vitro assessment of Ag and TiO_2 nanoparticles cytotoxicity

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Received: 22 July 2014
Accepted: 10 August 2014

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ABSTRACT

Background: Silver (Ag) and titanium dioxide (TiO_2) nanoparticles are the most eminent nanoproducts. Due to their antimicrobial and antifungal activity, they have been the well commercialized nanosubstances. The hazards associated with human exposure to Ag and TiO_2 nanoparticles should be investigated, and hence both the nanoparticles were synthesized to facilitate the risk assessment process.
Methods: Prior to the cytotoxic studies, Dynamic Light Scattering (DLS) and Transmission Electron Microscopy (TEM) were carried out to ensure the particle size. Glutathione (GSH), Nitric Oxide (NO) and superoxide dismutase (SOD) estimated by ELISA method.
Results: In the present study, the cytotoxicity of Ag and TiO_2 were investigated by using the glutathione (GSH), Nitric Oxide (NO) and superoxide dismutase (SOD) by incubating various concentration of silver (0.25 to 76 mg/mL) and titanium dioxide (0.25 to 2 mg/mL) nanoparticles in different incubation periods (24, 48 and 74 hours at 37°C) in plasma. Results observed that significant decrease ($P < 0.0001$) in the concentration of GSH associated with increased concentration of NO ($P < 0.0001$) and SOD ($P < 0.0001$) after incubation with silver and titanium dioxide nanoparticles at 24hrs at 37°C, however at 48 hours and 74 hours there is not much change.
Conclusion: The results indicate that silver and titanium dioxide nanoparticles exhibits, nanoparticles mediated cytotoxicity by induction of Reactive Oxygen Species (ROS).

Keywords: Nanoparticles, ROS, Cytotoxicity, GSH, NO, SOD, Silver, Titanium dioxide

INTRODUCTION

The nanosized Ag and TiO_2 particles engineered exhibit characteristics such as small size, large surface area to mass ratio, reactive surface groups etc. These properties offer great opportunity in the development of nanoparticle applications in various industries.[1,2] Despite the widespread applications of Ag and TiO_2 nanoparticles, there is still a lack of information concerning their toxicity at the cellular and molecular levels. Ag has been shown to directly interact with the cell membranes of bacteria causing breaks in the membrane and the cell to essentially burst.[3] It is this innate property that has led to an array of potential applications of Ag nanoparticles in both the medical arena and consumer products. Ag has traditionally been used as an antimicrobial agent[4] for many years in a diverse range of applications: air sanitizer sprays, socks, pillows, slippers, face masks, wet wipes, detergent, soap, shampoo, toothpaste, air filters, coatings of refrigerators, vacuum cleaners, washing machines, food storage containers, cellular phones, liquid condoms, wound

dressings, contraceptive devices, surgical instruments and bone prostheses which can be coated or embedded with the Ag nanoparticles.[5-8]

Titanium dioxide (TiO_2) nanoparticles are widely used in several manufactured products. TiO_2 particles with diameters larger than 100 nm are considered biologically inert in both humans and animals.[9] Based on this understanding, TiO_2 nanoparticles have been widely used in many products, such as white pigment, food colorant, sunscreens, tablets and cosmetic creams.[10] The potential adverse health effects due to the prolonged exposure at various concentration levels of these NPs in humans and environment has not yet been established.

It is very important to understand how Ag and TiO_2 nanoparticles interact with human body. Because of large surface area, Ag and TiO_2 nanoparticles are immediately adsorbed to macromolecules they encounter leading to genotoxicity and cytotoxicity by generating Reactive Oxygen Species (ROS). ROS form as a natural byproduct of the normal metabolism of oxygen and have important roles in cell signaling and homeostasis. However, during Ag and TiO_2 nanoparticles exposure, ROS levels can increase dramatically leading to cell damage and cell death.[11] To combat excess ROS, cells utilize antioxidants. If the equilibrium between ROS generation and the antioxidant defense within a cell is disrupted it may result in oxidative stress.[12] We subsequently measured NO and SOD levels to determine whether generation of reactive oxygen species could be a possible mechanism for the cytotoxicity observed for Ag and TiO_2 nanoparticles.

Major contributor to oxidative damage is hydrogen peroxide (H_2O_2), which is converted from superoxide that leaks from the mitochondria. Superoxide dismutase amends the damaging effects of H_2O_2 by converting into oxygen and water, benign molecules.[13] However, this conversion is not 100% efficient, and residual peroxides persist in the cell. The H_2O_2 produced by the action of NO and SOD is catalytically decomposed to water molecule by glutathione. GSH also scavenges other ROS molecules and prevents oxidation of protein sulfhydryl groups.[14] As reduced glutathione (GSH) has a central role in cellular antioxidant defense, we determined the effects of Ag and TiO_2 nanoparticles on changes in the intracellular glutathione content. Because of its importance (as an antioxidant), GSH is also a vulnerable target in cell defense, can directly deplete. Our study elucidates the cytotoxicity of Ag and TiO_2 nanoparticles by the quantification of GSH, NO and SOD in human plasma.

METHODS

Chemicals

Silver nitrate, sodium borohydride, 4-(prop-2-ynyloxy) pyridine, azide containing xylose, copper sulfate, ascorbic acid sodium, tetramethyl ammonium hydroxide, butanol and hydrochloric acid purchased from Sigma-Aldrich, India. Glutathione assay and super oxide assay kits procured from Cayman chemicals, USA, nitrotyrosine assay kit obtained from Hyeult Biotech and HPLC grade water purchased from Qualigens scientific, Mumbai.

Sample preparation

5 ml of whole blood was collected in EDTA coated vaccutainer. The blood was centrifuged at 700-1000x g for 10 minutes at 4°C. Plasma was collected and stored in refrigerator (-20°C) until use. The plasma was then incubated with various concentrations of Silver (Ag) and titanium dioxide (TiO_2) nanoparticles for 24, 48 and 72 hours at 37°C.

Synthesis of xylose modified silver nanoparticles

1.0 mL silver nitrate (10^{-2} mol/L) was added in 97 mL water and stirred for 5 minutes. Then 1.0 mL ice cold water containing 10 mg sodium borohydride was added followed by addition of 1.0 mL (10^{-3} mol/L) 4-(prop-2-ynyloxy) pyridine solution and the colloidal silver solution were stirred for 1h at room temperature. Finally, 4-(prop-2-ynyloxy) pyridine modified silver nanoparticle (PP-Ag NPs) were obtained. To PP-Ag NPs solution, 1.0 mL (10^{-3} mol/L) azide containing xylose derivative was added and stirred for 10 minutes. Then, to the above mixture, 0.1 mL mixture of copper sulfate (10^{-2} mol/L) and ascorbic acid sodium (10^{-3} mol/L) were added and heated for 2 hour at 60°C. Finally, the synthesized xylose modified silver nanoparticles (TX-Ag NPs) were purified by Amicon centrifugation. Ag-NPs were characterized using transmission electron microscopy (TEM) and dynamic light scattering (DLS) (Figure 1 & 2).

Synthesis of TiO_2 nanoparticles

The TiO_2 nanoparticles were synthesized by a solvothermal method.[5,15] In a 250 ml flask 257 mg of tetramethyl ammonium hydroxide was dissolved in 150 ml of water. The solution temperature was lowered down to 2°C by keeping the flask in an ice bath, and then 10 ml of (0.335 mmol) Titanium butoxide in butanol solution was added to it. The reaction mixture was then refluxed at 95°C for about 6 hr. The reaction mixture (50 ml) was transferred to an autoclave of 75 ml capacity and heated in an oven for 5 hour at 180°C. Thus obtained material was centrifuged, washed with Millipore water for 4 to 5 times and re-dispersed in water. A final concentration of 2 mg/ml for the TiO_2 dispersion was maintained at pH=2 by adding required amount of Hydrochloric acid (1M). The synthesized TiO_2 nanoparticle was subjected to an extensive characterization process X-ray diffraction analysis (XRD) and Dynamic Light Scattering analysis (DLS). XRD analysis was performed using Seimens (Chesir.UK) D5000 X-ray diffract meter and DLS, as-prepared TiO_2 dispersion in water at pH=2. The analysis was done with Malvern Zetasizer (Figure 3 & 4).

Effect of TiO_2 and Ag-NPs

Measurement of glutathione

The changes in GSH level were determined after incubating Ag and TiO_2-NPs in control peripheral plasma samples by using a commercially available kit (Cayman Chemicals, USA). In brief, Ag (0.25 to 76 mg/mL) and TiO_2 (0.25 to 2 mg/mL) nanoparticles incubated for 24 to 72 hours in plasma. After incubation plasma (50 µL) and standard (50 µl) were transferred to respective wells in 96-well plate, and 150 µl freshly prepared reagents and enzyme mixture were added and the absorbance was read after 25 min at 405 nm using micro plate reader. GSH activity expressed in U/mL.

Measurement of superoxide dismutase

The activity of superoxide dismutase was determined after incubating Ag and TiO_2-NPs in control plasma samples by using a commercially available kit (Cayman Chemicals, USA). In brief, Ag (0.25 to 76 mg/mL) and TiO_2 (0.25 to 2 mg/mL) nanoparticles incubated for 24 to 72 hours in plasma. After incubation plasma samples (10 µL) and standard (10 µL) were transferred to respective wells in 96-well plate, and 200 µL freshly prepared reagents and enzyme mixture were added and the absorbance was read after 20 minutes at 440 nm. SOD activity was expressed in U/mL.

Measurement of nitric oxide (NO)

Nitric oxide levels were quantified after incubating Ag and TiO_2-NPs in control plasma by using a commercially available kit (Cayman Chemicals, USA). In brief, Ag (0.25 to 76 mg/mL) and TiO_2 (0.25 to 2 mg/mL) nanoparticles incubated for 24 to 72 hours in plasma samples. After incubation plasma samples (100 µL) and standard (100 µL) were transferred to respective wells in 96-well plate, and the plate was incubated for 1 hour at 25°C, followed few washing steps, the absorbance was read at 450 nm. The SOD activity was expressed in U/mL.

Statistical analysis

Statistical Analysis was performed using Sigma Stat. Software (SPSS, Inc., Chicago, IL). Data were expressed as mean ± SD for the values obtained from at least three independent experiments. Statistical analysis was performed by one-simple t-test. The level of statistical significance chosen was $P < 0.05$.

RESULTS

XRD and TEM analysis of Ag and TiO_2-NPs

The typical TEM image shown in Figure 1 clearly suggests that most of the Ag nanoparticles are spherical in shape. The X-ray diffraction pattern of TiO_2-NPs obtained by solvothermal method is shown in Figure 3. The peaks were indexed using Powder X-ray diffraction and were found corresponding with the anatase structure of TiO_2. No impurity phase was observed in the sample. The average crystallite size of the samples was calculated using Debye Scherrer's formula. The estimated crystallite size corresponding to the most intense crystallographic plane (101) was determined to be 12 nm.

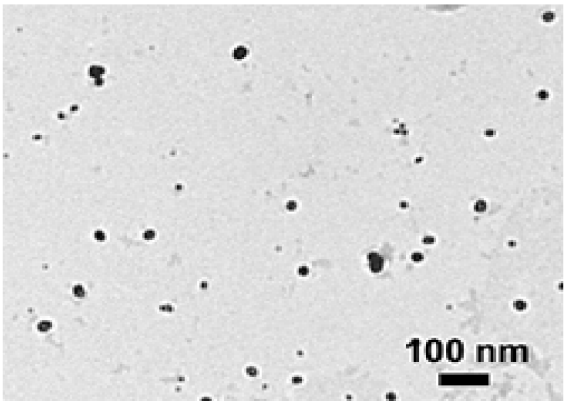

Figure 1: TEM image of xylose-modified silver nanoparticles.

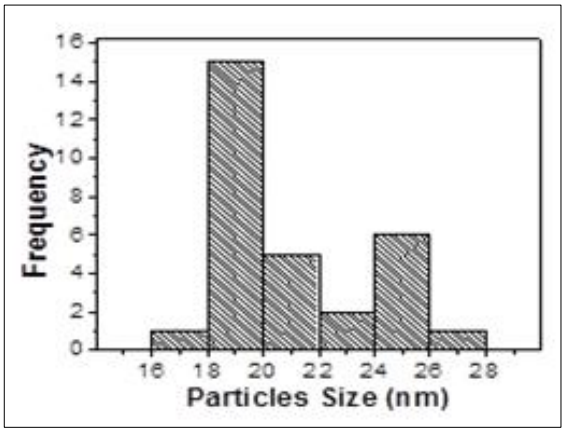

Figure 2: DLS image particle size distribution of xylose-modified silver nanoparticles.

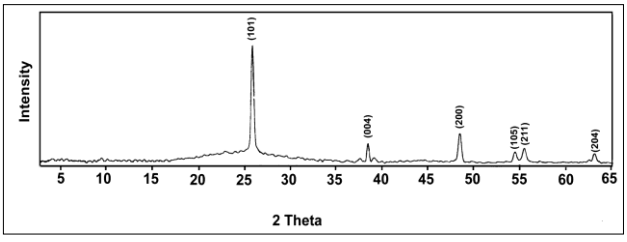

Figure 3: X-Ray diffraction (XRD) pattern for TiO_2 sample. XRD analysis was performed using Seimens (Chesir.UK) D5000 X-Ray diffract meter, CuKα (λ=1.5406°A) as monochromatic radiation source. The XRD pattern could be indexed to an anatase phase of TiO_2 (JCPDS 71-1167, 71-1166).

Dynamic light scattering analysis of Ag and TiO_2-NPs

The DLS result shows that the Ag nanoparticles have a size distribution of 16-28 nm, with a maxima centred at 19 nm (Figure 2). Figure 4 shows the dynamic light scattering analysis of TiO_2-NPs. The hydrodynamic size was found to be in range of 15-50 nm.

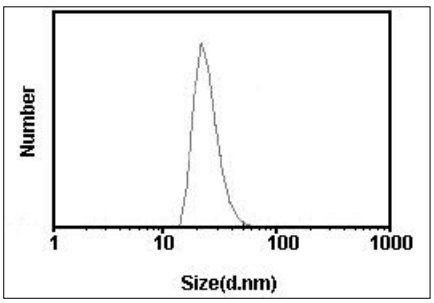

Figure 4: Dynamic light scattering analysis of as-prepared TiO_2 dispersion in water at pH=2. Analysis was done with Malvern Zetasizer with measurement of back scattered angle at 175°.

GSH levels of Ag and TiO_2-NPs induced were measured to assess the extent of oxidative stress in the plasma

The Ag and TiO_2-NPs induced ROS generation in plasma was analyzed by Enzyme Linked Immuno Sorbent Assay (ELISA). The ability of Ag and TiO_2 nanoparticles to induce oxidative stress was evaluated by measuring reactive oxygen species, glutathione, superoxide dismutase, and NO levels on human plasma. The results show that Ag and TiO_2 nanoparticles induced generation of intracellular reactive oxygen species in a dose-dependent manner (Table 1 & 2).

A standard graph was plotted for reduced glutathione (GSH) by taking concentration of GSH on X-axis and absorbance at 414 nm on Y-axis from which the concentration of GSH from Ag and TiO_2 nanoparticles induced plasma sample was calculated. The results showed that there was decrease in the concentration of GSH when compared to the control sample (human plasma without addition of Ag and TiO_2 nanoparticles) in all the three incubation periods 24, 48 and 72 hours ((Table 1 & 2; Figure 5).

Table 1: Effect of Ag NPs at various concentrations on glutathione, SOD and NO at 24, 48, 76 hours incubation at 37°C.

Conc. of Ag NPs (mg/L)	Biochemical parameters	Incubation at 37°C for 24 hours	Incubation at 37°C for 48 hours	Incubation at 37°C for 72 hours
Control	Glutathione (µM)	3.22	3.21	3.25
	SOD (U/ml)	0.45	0.45	0.46
	NO (µM)	0.4	0.4	0.4
0.25	Glutathione (µM)	2.0	2.55	2.55*
	SOD (U/ml)	0.27	0.17**	0.25*
	NO (µM)	7.4**	1.6	0.2*
0.5	Glutathione (µM)	2	2	2.2*
	SOD (U/ml)	0.25	0.19	0.20*
	NO (µM)	10.6**	1.7	0.2
1	Glutathione (µM)	2.35	2.25	2.8 *
	SOD (U/ml)	0.15**	0.19	0.24
	NO (µM)	7.3	1.1	0.2
2.5	Glutathione (µM)	1.8 **	1.7	2.25
	SOD (U/ml)	0.27	0.27*	0.42
	NO (µM)	11.2**	1.3	0.2
5	Glutathione (µM)	1.7	1.5 **	2.25
	SOD (U/ml)	0.24	0.16**	0.31
	NO (µM)	8.6**	1.4	0.2
7.6	Glutathione (µM)	1.5*	2.2	2.25
	SOD (U/ml)	0.30	0.19	0.27*
	NO (µM)	11.85**	1.7	0.2
76	Glutathione (µM)	1.8	2	1.7*
	SOD (U/ml)	0.25	0.23*	0.31
	NO (µM)	9.25*	2	0.2**

Data represent the mean ± SD of three independent experiments done in duplicate.
*P <0.001, **P <000.1

Table 2: Effect of TiO_2 NPs at various concentrations on Glutathione, SOD and NO at 24, 48 and 76 hours incubation at 37°C.

Conc. of TiO2 NPs (mg/mL)	Biochemical parameters	Incubation at 37°C for 24 hours	Incubation at 37°C for 48 hours	Incubation at 37°C for 72 hours
Control	Glutathione (µM)	3.2	3.2	3.2
	SOD (U/ml)	0.46	0.45	0.46
	NO (µM)	0.5	0.4	0.4
0.25*	Glutathione (µM)	3.2	2.85	2.2*
	SOD (U/ml)	0.20**	0.24**	0.27**
	NO (µM)	7.2**	1.4**	0.5*
0.5*	Glutathione (µM)	2.7	2.55*	2.7*
	SOD (U/ml)	0.34	0.19**	0.34
	NO (µM)	7.4**	1.4**	0.1**
1*	Glutathione (µM)	2.55*	2.5*	2.55*
	SOD (U/ml)	0.30*	0.19**	0.27*
	NO (µM)	7.4**	2.9**	0.4
1.5*	Glutathione (µM)	2.45*	2.55*	2.7*
	SOD (U/ml)	0.25**	0.20**	0.37
	NO (µM)	7**	1.8**	0.7*
2*	Glutathione (µM)	3.0	2.80	2.55*
	SOD (U/ml)	0.25**	0.20**	0.25**
	NO (µM)	7**	1.9**	0.4

Data represent the mean ± SD of three independent experiments done in duplicate.
*P <0.05

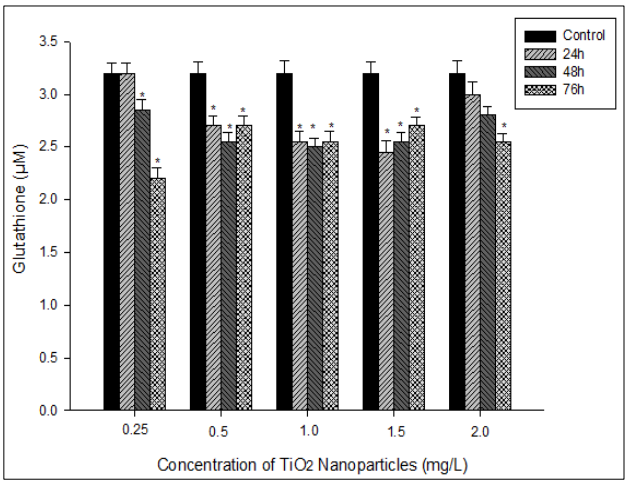

Figure 5: Effect of TiO2-NPs on Glutathione levels in plasma exposed to 24h, 48h and 76h at 37°C. Each histogram represents the mean ± SD of three independent experiments. *P <0.001.

A standard graph was plotted for Super Oxide Dismutase (SOD) by taking concentration of SOD on X-axis and absorbance at 440 nm on Y-axis from which the concentration of SOD from Ag and TiO_2 nanoparticles induced plasma sample was calculated. Results showed that there was increase in the SOD activity in all the three incubation periods 24, 48 and 72 hours when compared to the control human plasma samples (Table 1 & 2).

The concentration of NO was calculated using the standard graph (concentration of NO on X-axis and absorbance at 450 nm on Y-axis, Fig not included), which showed increase in the concentration of NO in Ag and TiO_2 nanoparticles incubated 24 hours sample. But in 48 and 72 hours sample the concentration of NO decreased drastically (Table 1 & 2; Figure 6).

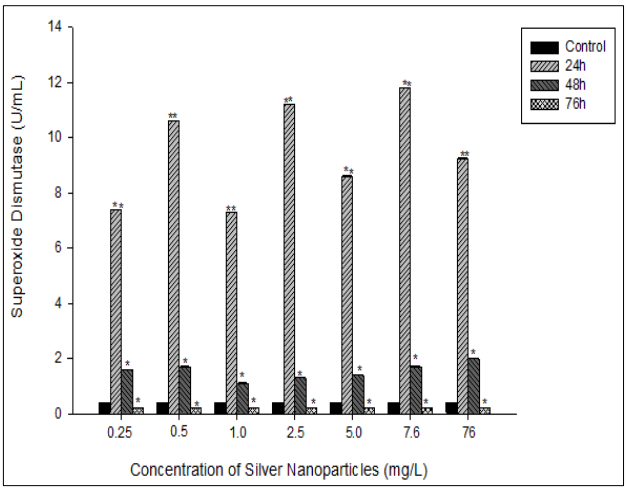

Figure 6: Effect of Ag-NPs on Nitric Oxide levels in plasma exposed to 24h, 48h and 76h at 37°C. Each histogram represents the mean ± SD of three independent experiments. *P <0.001, **P <0.0001.

DISCUSSION

Our aim of this study was to evaluate potential toxicity involved in nanoparticles-induced cytotoxicity and the general mechanism. In this study, the various concentrations of Ag and TiO_2-NPs induced cytotoxicity were investigated in human plasma.

In our study it was observed that the exposure of Ag-NPs at dosage levels of 0.25 to 76 mg/L exhibited cytotoxicity in dose and time dependent manner and it was revealed by glutathione, SOD and nitric oxide assay. It is generally observed that more the NPs concentration and greater the toxicity.[13,16] It was evident that cellular oxidative stress was exhibited may be due to elevated NO and SOD levels with reduced GSH, which indicates that ROS induced cytotoxicity by damaging mitochondrial cells. There was strong correlation between decreased GSH and increased NO and SOD levels after 24 hours of incubations. This study supported by study conducted by Quaiser Saquib et al.[17] Few of the studies showed NP mediated cytotoxicity, which includes oxidative stress, genetic damage, inflammation and the cell death.[18-21]

Like nanoorganisms (viruses), nanoparticles are able to enter cells and interact with subcellular structures. Cellular uptake, subcellular localization, and ability to catalyze oxidative products depend on nanoparticle chemistry, size, and shape.[10] The mechanism by which nanoparticles penetrate cells without specific receptors on their outer surface is assumed to be a passive uptake or adhesive interaction. This uptake may be initiated by van der Waals forces, electrostatic charges, steric interactions, or interfacial tension effects, and does not result in the formation of vesicles.[22,23] The synthesis and applications of metal oxide NPs are consistently expanding due to their distinctive physico-chemical characteristics, and increased industrial and medical applications. This has evoked serious concerns about their potential impact on the environment and human health. Due to their small aerodynamic diameter, the ultrafine particles (<100 nm) from natural and anthropogenic sources including viruses, biogenic magnetite, ferritin, metal oxides, fullerenes, carbon, polymers and other fumes may contaminate ambient air, penetrate deep into the lungs, and reach different body organs through the blood circulatory system.[10,11,24]

The common tendency of TiO_2-NPs to readily diffuse through the protective cellular barriers may also involve risks to human health, and warrants systematic and in-depth investigation of their possible toxicological effects. Therefore, in this study, we have determined the cytotoxicity of crystalline Ag and TiO_2-NPs using human plasma for understanding the NPs toxicity. Human plasma cells have been used in earlier studies to assess the oxidative stress.[25-27] The synthesized and well characterized polyhedral rutile Ag and TiO_2-NPs, suspended in RPMI medium were used for cellular treatment and toxicity assessment in plasma. Since, various concentration of the NPs are regarded as important parameters for in vitro cytotoxicity in a cell culture medium, therefore, the behavior of Ag and TiO_2-NPs in cell culture medium was evaluated through Dynamic Light Scattering (DLS), to understand the extent of aggregation of these NPs before cellular exposure. DLS is widely used to determine the size of Brownian NPs in colloidal suspensions in the nano and submicron ranges [15, 28]. The presence of variable sized (10 to 40 nm) TiO_2-NPs aggregates in RPMI culture medium corroborates well with the earlier reports.[29-31] Thus, the DLS data revealed the formation of Ag and TiO_2-NPs aggregates in the RPMI cell culture medium, which were also found to be internalized in the TEM images of the treated cells.

Assessment of Ag and TiO_2-NPs cytotoxicity through the GSH, NO and SOD assays exhibited the dose dependent toxic effects on cell viability in a concentration range of silver (0.25 to 76 mg/mL) and titanium dioxide (0.25 to 2 mg/mL) nanoparticles with various incubation timings (24h, 48h and 76h at 37°C). There was a significant decrease in the concentration of GSH associated with increased concentration of NO and SOD after incubation with silver and titanium dioxide nanoparticles at 24 hours at 37°C, however at 48 hours and 74 hours significant changes were not observed.

Silver and TiO_2-NPs induced changes at the levels of oxidative markers (GSH, SOD and NO), intracellular ROS generation and consequent cell toxicity. The data revealed significant depletion in GSH level in Ag and TiO_2-NPs treated plasma cells at 76 mg/mL and 2 mg/mL, as compared to the untreated control. The results support the study of Nemmar et al. (2011) on the rutile Fe-doped TiO_2 nano rods induced dose dependent decrease in the SOD and GSH levels in the hepatic and heart tissues of rats.[32] The results also correlate the earlier reports on TiO_2-NPs induced ROS production in bronchial epithelial cell line (BEAS-2B) at 10 mg/mL concentration.[9] Also, significant intracellular ROS production has been demonstrated in the human epidermal (A431) and brain microglia (mouse BV2) cells at TiO_2-NPs doses of 80 mg/mL and 25 mg/mL, respectively.[33,34] Thus, the dose comparison of our study with earlier reports suggests the induction of oxidative stress in the plasma at relatively lesser concentrations of TiO_2-NPs. Surface reactivity has been suggested to plays an important role in ROS production by NPs.[1,35] Significant nano toxicity exhibited at 2 mg/mL ($P < 0.001$) and 76 mg/mL ($P < 0.0001$) concentrations of Ag and TiO_2-NPs signifies that the primary mechanism of NPs induced toxicity is due to oxidative stress, resulting in damage to cellular membranes and biological macromolecules, as reported earlier.[10,24,34] Our previous reported results have also demonstrated that the TiO_2-NPs induce DNA damage at a critical concentration of 5 mg/mL.[36] The data suggests that the TiO_2-NPs at lower concentrations up to 5mg/mL modulate the antioxidant enzymes levels, whereas, at higher concentrations, the

cellular DNA repair machinery may be adversely affected. Thus, the degree of induced cytotoxicity effect, and risk assessment of the nonmaterial and nanoproducts should be assessed prior to their larger applications in spite of their apparent extraordinary advantages.

CONCLUSION

We have concluded our findings in this study for the first time explicitly demonstrated the cytotoxicity of Ag and TiO_2-NPs in human plasma. Significant reduction in marker antioxidant levels suggested their role in inducing oxidative stress leading to cell death. It is contemplated that the differential susceptibility of cell types could be due to differences in their metabolic rate, antioxidant enzyme machinery, and DNA repair capabilities, which may exhibit variability in Ag and TiO_2-NPs induced toxic effects on human health.

ACKNOWLEDGEMENTS

We thank Kamineni institute of medical sciences for constant encouragement and support to publish this work.

Funding: The study was funded by the department of biotechnology, New Delhi (Grant sanction order ref No.: BT/PR15094/GBD/27/313/2011)
Conflict of interest: None declared
Ethical approval: The study was approved by the human ethical clearance committee of Kamineni institute of medical sciences

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DOI: 10.5455/2320-6012.ijrms20141122
Cite this article as: Ganapathi AP, Devaki R, Thuniki NR, Manna J, Tirumuru B, Gopu CR, Deepthi SB, Trivedi R, Rana RK, Hasan A. *In vitro* assessment of Ag and TiO_2 nanoparticles cytotoxicity. Int J Res Med Sci 2014;2:1360-7.