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An *in vitro* study on the induction of micronuclei and other nuclear anomalies in peripheral blood lymphocyte culture by metal oxide nanoparticles

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Abstract

Increase in the production and release of engineered nanoparticles are known to induce genetic alterations that directly and indirectly affect the human health. The present study was aimed to evaluate the genotoxic potential of two metal oxide nanoparticles, silicon dioxide (SiO_2-NPs) and titanium dioxide (TiO_2-NPs), in human peripheral lymphocyte *in vitro*. Human peripheral blood cells were cultured with two different concentrations (0.5 µg/mL and 1µg/mL) of SiO_2 and TiO_2 and the genotoxicity was measured using CBMN (Cytokinesis Block Micronucleus) assay. Binucleated cells with micronuclei (Mni), nucleoplasmic bridges (NPBs) and nuclear buds (Nbuds) were scored along with multi- and mono-nucleated cells. The results showed moderate to highly significant frequency of genotoxicity when compared with untreated negative control. The total nuclear anomalies by using cytokinesis block proliferation index were found to be increased at 10-15 folds in the treated samples than that of the control. The present study shows that the metal oxide nanoparticles induce the formation of micronuclei and other nuclear anomalies in peripheral blood lymphocytes *in vitro*.

Keywords: Nanoparticles, Genotoxicity, CBMN assay, Micronucleus, Nuclear anomalies.

Introduction

Recently engineered nanoscale particles or engineered nanoparticles are enormously produced and released into the environment. The toxicological or biological effects of such nanoparticles are studied based on the nature and mechanism of action on biological systems or living organisms. Nanotoxicology is one of the growing fields of science that aim to concentrate on the toxicological effects of nanoparticles that pose threat to the ecosystem and human health. The present study focused mainly on two metal oxide nanoparticles such as silicon dioxide (SiO_2-NPs) and titanium dioxide (TiO_2-NPs). They are largely introduced into the environment intentionally or by accidental spillage by human. TiO_2 nanoparticles are widely used in consumer products, biomedical applications, electronic and optic devices, and also as a photocatalyst in air and water remediation (Handy and Shaw, 2007). SiO_2 nanoparticles are extensively used nanomaterials developed for a broad spectrum of biomedical and biotechnological applications such as biosensors for DNA, cancer therapy, gene delivery, drug delivery, in industrial manufacturing, packaging and ceramic synthesis (O'Farrell *et al*., 2006). Thus all living beings are continuously exposed to such nanoparticles, which may pose high threat, particularly to human health.

Most of the nanoparticles possess unique physico-chemical properties that are capable to get bioaccumulate inside the cell. It can also interact with genetic material and induce genotoxicity, which could be both chromosomal aberrations and DNA damages. For genotoxicity studies, *in vitro* methods are usually employed where cytokinesis block micronucleus assay (CBMN) is the most reliable method for evaluating genotoxicity. Nanoparticles are suspected to be genotoxic as they are capable to induce reactive oxygen species generation thereby leads to oxidative stress mediated DNA damage in cell line models (Di Virgilio *et al*., 2010; Magdolenova *et al*., 2012; Kazimirova *et al*., 2012). Several literatures showed that in cultured cells, nanoparticles interrupt cellular functions by inducing oxidative stress, DNA damages and also altered cell signalling and cell cycle pathways (Donaldson *et al*., 2002; Wang *et al*., 2007). The available data is inadequate to predict and develop the concept of biologically safe dose of nanoparticles as its effects on different targeted cells depends not only on the dose, duration and endpoints but also based on the chemical composition, size, surface properties, structure etc. Some of the existing information on genotoxic potential of nanoparticles is highly contradictory and inconclusive. For instance, exposure to nano silica showed negative genotoxicity by using alkaline comet assay whereas by micronucleus assay it showed positive genotoxicity (Rim *et al*., 2013). The most widely studied metal oxide nanoparticles are SiO_2 and TiO_2 as its occupational and industrial exposure is comparatively high, but still the establishment of a standard procedure for testing nano genotoxicity remains scanty. In this study, an attempt was made to investigate the genotoxic effects of the selected metal oxide nanoparticles in peripheral blood lymphocytes.

Materials and Methods

Chemicals

TiO_2-NPs (Cat. No: 634662; Titanium (IV) oxide, mix of anatase and rutile) were obtained from Sigma Aldrich, Germany. SiO_2-NPs (Cat. No: 1940323) was obtained from SISCO Research Laboratory (SRL), India. The purity and size of the nanoparticles are further confirmed by X-ray diffraction and Transmission Electron Microscopy. The nanodispersions of TiO_2 and SiO_2 were prepared just before exposure by ultra sonication at 100 kHz for 30 min and 10 min, respectively using double distilled water and maintained as stock. All other chemicals were of analytical grade and were obtained from local commercial sources.

Cytokinesis Block Micronucleus Assay

The cytokinesis-block micronucleus (CBMN) assay was done according to the method as described by Fenech (2007). For this, 7.5 mL of peripheral blood from donor was drawn to make triplicates of each treatment group. Blood cell cultures were set up by mixing 0.5 mL of whole blood with 4.5 mL of Hikaryo XL (HiMedia) ready mix media. The Cytokinesis Block Micronucleus assay was performed by adding the test agents. In Group I, 0.5 µg/mL and 1µg/mL concentrations of SiO_2 and in Group II, 0.5 µg/mL and 1µg/mL concentrations of TiO_2 were treated with the peripheral blood cell culture at 44 h and cytochalasin B was added at 48 h to explore the frequency of genotoxic events in the treated and untreated cultures. Harvesting was carried out at the end of 72 h where the cells were treated hypotonically with cold 0.075 M KCl for 8 min, followed by fixation with methanol-acetic acid (3:1). Subsequently, slides were prepared and stained with Giemsa solution (4%). For each sample, cells were scored for anomalies such as micronucleus (MNi), nucleoplasmic bridges (NPBs) and nuclear buds (Nbuds) (Fenech *et al*., 2003; 2007).

Cytokinesis Block Proliferation index (CBPI)

CBPI was calculated according to the method as described by Eastmond and Tucker (1989)
CBPI is based on the formula:

$$CBPI = [MI + 2MII + 3 (MIII + MIV)]/[MI + MII + (MIII + MIV)]$$

Where:
MI -Mononucleate cell
MII- Binucleate Cell
MIII- Trinucleate cell
MIV -Tetranucleate Cell

Scoring for nuclear abnormalities

DNA damage is scored specifically in once divided binucleated cells and includes micronuclei (MNi), nucleoplasmic bridges (NPBs), and nuclear buds (Nbuds). A minimum of 1000 binucleated cells were scored (Fenech *et al*., 2003). CPBI was calculated using the frequencies of mono-, bi-, tri- and tetra nucleated cells obtained/500 cells. Microphotographs of selected nuclear anomalies were taken using Carl Zeiss Axioscope 1.

Statistical analysis

Statistical analysis of the data was performed using Graph Pad Prism (version 6.0). Students t-test was used to determine the statistical significance where $P<0.05$ is set significant against the control samples.

Results

Cytokinesis Block Proliferation index (CBPI)

There was a significant ($p<0.05$) increase in the cytokinesis block proliferation index of SiO_2 and TiO_2 treated cultures when compared with untreated culture (Figure 1). Both cultures containing SiO_2 and TiO_2 nanoparticles at both the concentrations (0.5 µg/mL and 1 µg/mL) showed a dose-dependent increase in the proliferation index of human peripheral blood lymphocyte culture.

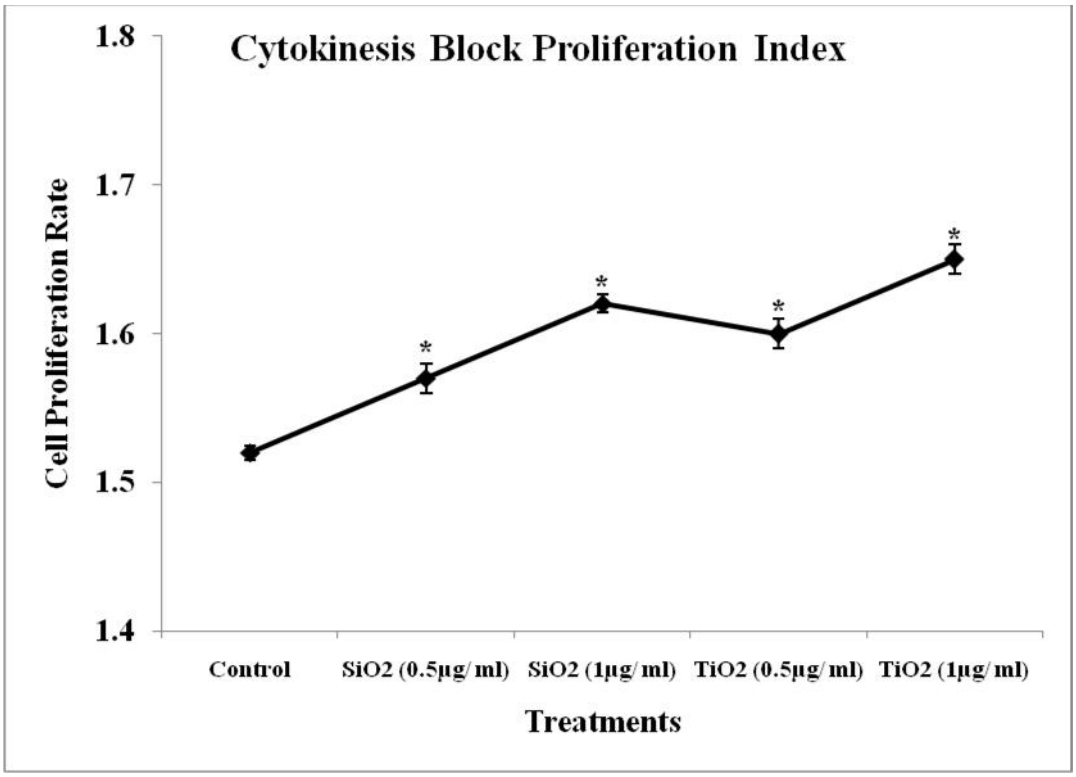

Figure 1: Cytokinesis block proliferation index in the control and treated human peripheral blood lymphocyte cultures

Nuclear anomalies

SiO_2 and TiO_2 when cultured with human peripheral blood lymphocytes showed nuclear anomalies like micronuclei, nucleoplasmic bridge and nuclear buds (Table 1; Figure 6). The total nuclear anomalies showed 10 to 15 fold increase in the treated samples when compared to the untreated cultures. At both concentrations of SiO_2 and TiO_2, a significant ($P<0.05$) increase in total nuclear anomalies was observed (Figure 2). The frequency of micronuclei also increased in treated groups when compared with the untreated groups (Figure 3). However, there is no dose-dependent increase in the frequency of nucleoplasmic bridges in SiO_2 treated cultures, whereas TiO_2 treatment showed a dose-dependent increase in the frequency of nucleoplasmic bridges (Figure 4). Nuclear buds were increased at both concentrations of SiO_2 and TiO_2 treated cultures in dose-dependent manner (Figure 5).

Table 1: Nuclear abnormalities in SiO_2 and TiO_2 nanoparticles cultured with human peripheral blood lymphocytes. Asterisks (*) indicates the level of significance

Parameter	Treatment	Mean ± SEM	P<0.05
Mni	Control	2.000 ± 1.155	
	SiO2-(0.5 µg/mL)	18.67 ± 0.6667	0.0002***
	SiO2-(1 µg/mL)	20.00 ± 2.082	0.0016**
	TiO2-(0.5 µg/mL)	21.67 ± 1.202	0.0003***
	TiO2-1 (1 µg/mL)	20.67 ± 1.453	0.0005***
NPB	Control	0.3333 ± 0.3333	
	SiO2-(0.5 µg/mL)	2.667 ± 0.8819	0.0686
	SiO2-(1 µg/mL)	2.000 ± 0.5774	0.0668
	TiO2-(0.5 µg/mL)	1.667 ± 0.3333	0.0474*
	TiO2-1 (1 µg/mL)	3.000 ± 0.5774	0.0161*
NBUD	Control	0.6667 ± 0.6667	
	SiO2-(0.5 µg/mL)	11.33 ± 1.202	0.0015**
	SiO2-(1 µg/mL)	13.67 ± 1.856	0.0027**
	TiO2-(0.5 µg/mL)	8.667 ± 1.202	0.0043**
	TiO2-1 (1 µg/mL)	17.00 ± 0.5774	0.0001***
Total NA	Control	3.000 ± 1.732	
	SiO2-(0.5 µg/mL)	32.67 ± 2.404	0.0006***
	SiO2-(1 µg/mL)	35.67 ± 1.202	0.0001***
	TiO2-(0.5 µg/mL)	32.00 ± 1.528	0.0002***
	TiO2-1 (1 µg/mL)	40.67 ± 1.856	0.0001***

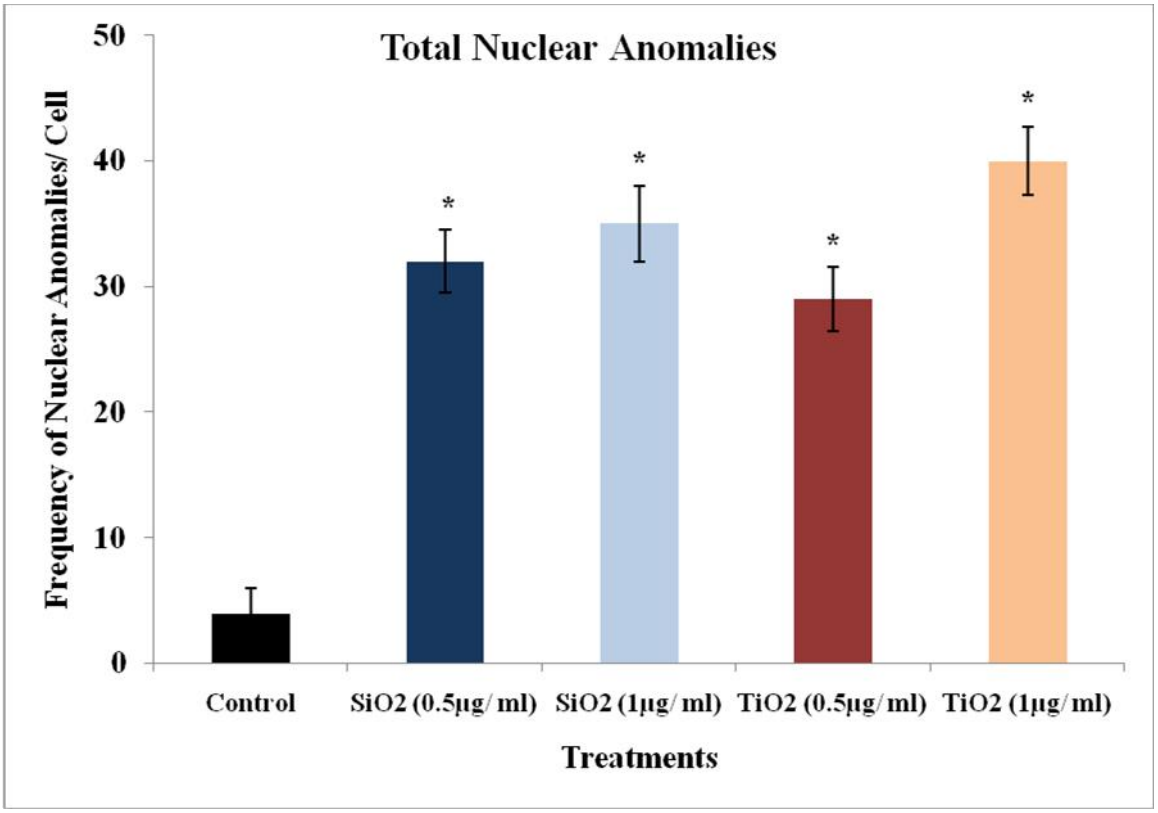

Figure 2: Total nuclear anomalies in SiO_2 and TiO_2 treated cultures

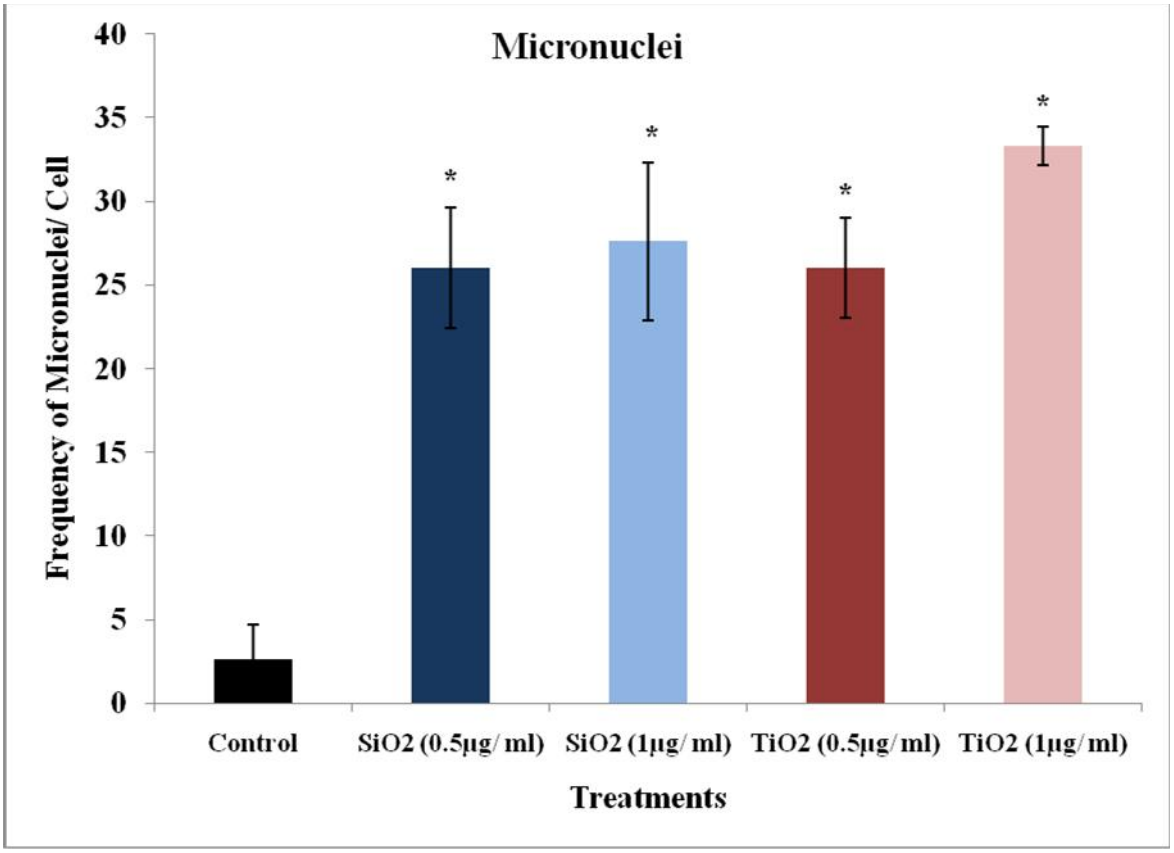

Figure 3: Frequency of micronuclei in SiO_2 and TiO_2 treated cultures

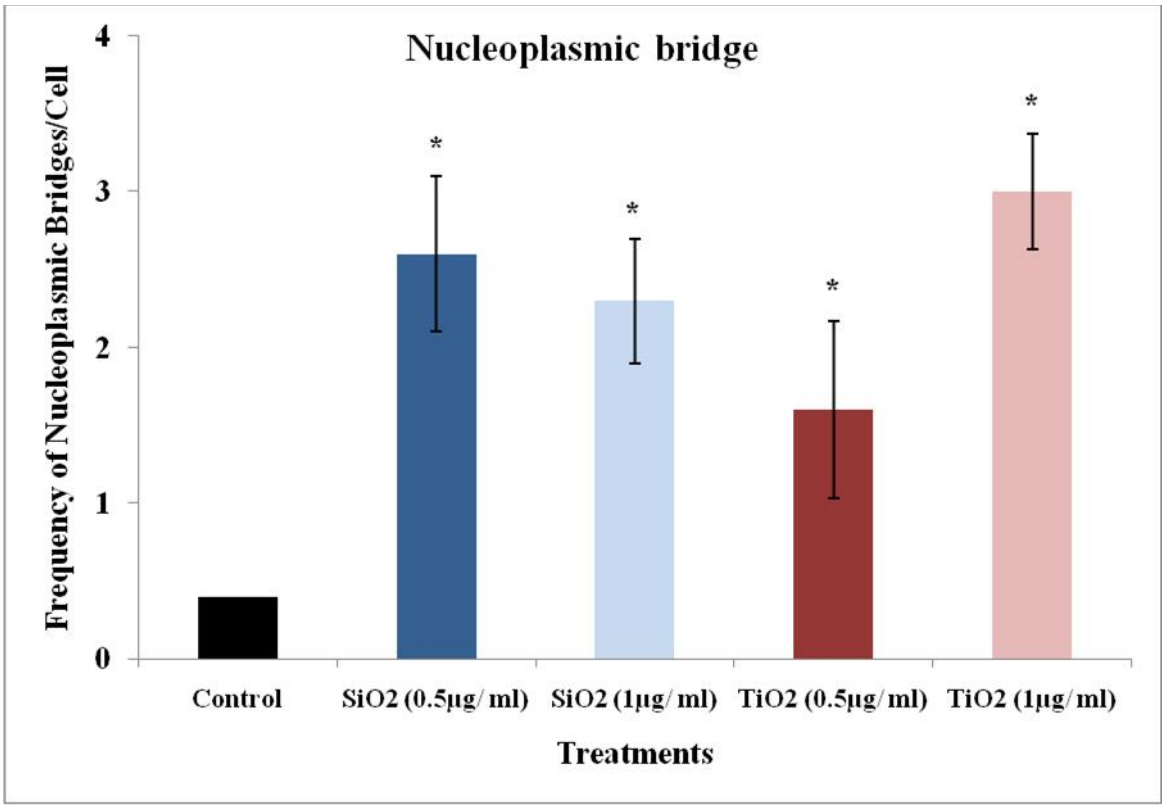

Figure 4: Nucleoplasmic bridges in SiO_2 and TiO_2 treated lymphocyte cultures

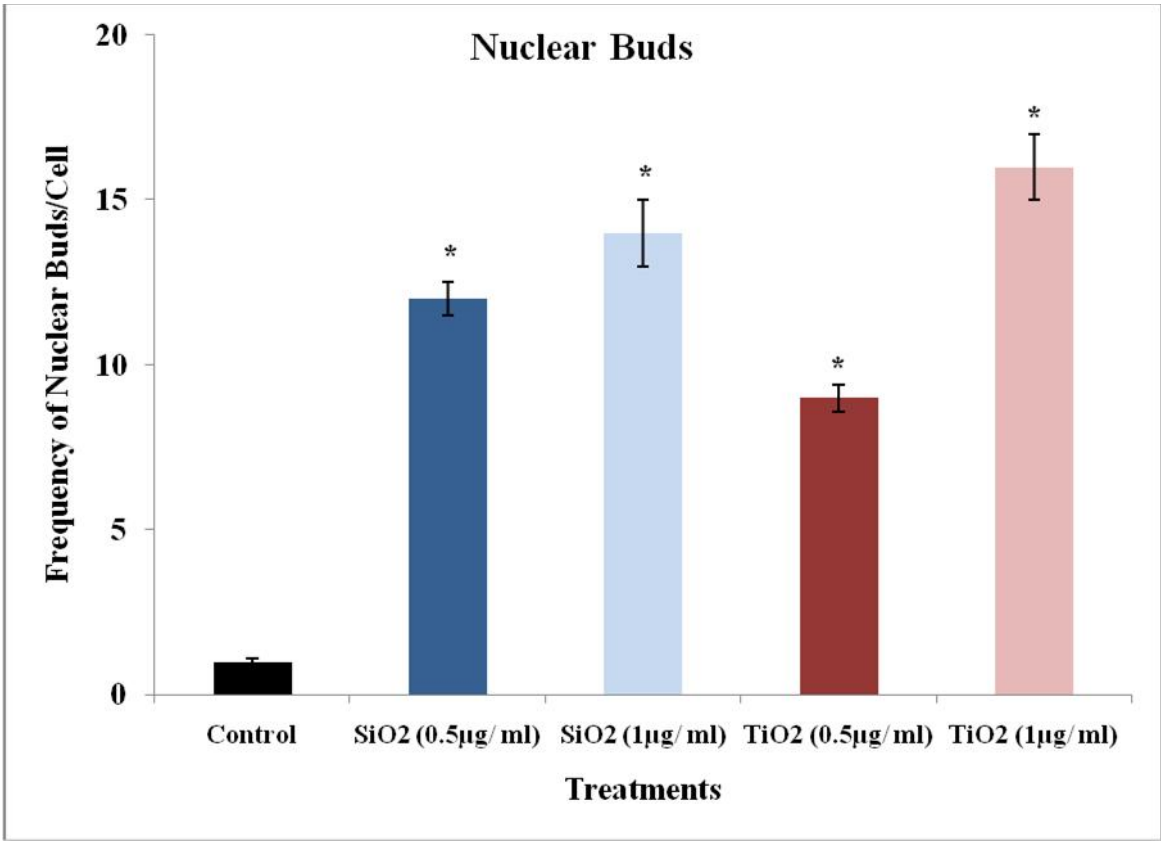

Figure 5: Frequency of nuclear buds in SiO_2 and TiO_2 treated cultures

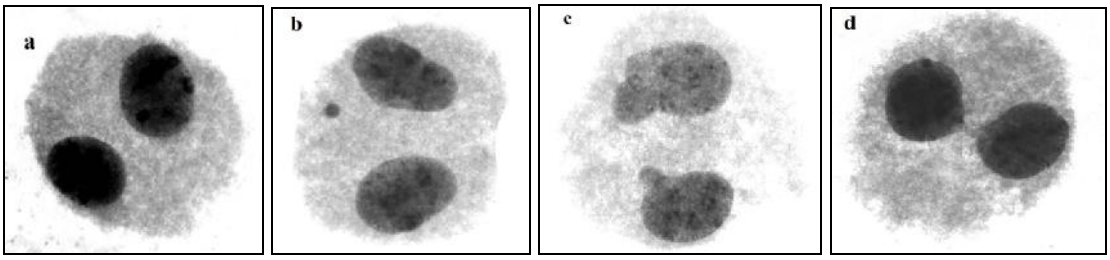

Figure 6: a. Binucleated cell; b. Micronucleus; c. Nuclear bud; d. Nucleoplasmic bridge

Discussion

Genotoxic effects of SiO_2 and TiO_2 nanoparticles at 0.5µg/mL and 1µg/mL concentrations were assessed in the human peripheral blood lymphocyte culture. All the treatment groups showed positive genotoxicity when compared to the control group. The major nuclear anomalies like proliferation frequency, micronuclei, nucleoplasmic bridges and nuclear buds were prominent. Nuclear proliferation index or nuclear division index gives the information regarding the cytostatic and cytotoxic properties of test chemical. For this mononucleated to multinucleated cells were scored and evaluated. Higher concentrations of both SiO_2 and TiO_2 showed increased proliferation index which states that the nanoparticles have a cytotoxic effect. Frequency of micronuclei was found to increase in all treatment groups. Micronuclei are formed as a result of breakage and loss of DNA or chromosome that lag behind at anaphase of the cell cycle (Fenech, 2007). Once divided cells are only scored to avoid the confounding effects caused by the altered cell cycle kinetics and it is made possible by scoring only the micronuclei in binucleated cells. Nucleoplasmic bridges and nuclear buds are usually the indications of dicentric chromosome and gene amplification, respectively. As the Cytokinesis is blocked, anaphase dicentric chromosome leads to the formation of bridges (Fenech, 2007).

CBMN method is the most reliable and efficient assay whose interpretations can be directly or indirectly give the measurements of cellular and DNA damage. The formation of micronuclei could be due to the unrepaired chromosome breaks, DNA misrepair, acentric chromososome fragments, asymmetrical chromosome rearrangement, malaggregation of chromosomes or defective cell cycle checkpoints, chromosomal instability etc. Likewise, the nucleoplasmic bridges are the result of telomere end fusions, breakage-fusion-bridge cycle etc and the mechanisms involved in nuclear bud formation are nuclear elimination of amplified DNA, chromosomal instability and the elimination of DNA repair complex (Fenech *et al*., 2003; 2007). TiO_2 is the mixture of two forms such as, anatase and rutile, and among them anatase is more toxic than rutile in cell cultures (Magdolenova *et al*., 2012). Therefore, the mechanism of TiO_2 induced genotoxicity may be more complex. In the present study TiO_2 nanoparticles showed more positive genotoxicity than SiO_2 nanoparticles and this could be due to its peculiar structure and surface properties that increased internalization of TiO_2-NP which could directly affect the nucleus. However, SiO_2 nanoparticles also showed positive genotoxicity where SiO_2 is hydrophilic nanoparticles and in animal model studies, SiO_2 has been shown to induce reactive oxygen species generation (Ramesh *et al*., 2012; Vidya *et al*., 2015). So oxidative stress induced DNA damage and oxidation of nuclear molecules could be the mechanism of the genotoxicity induced by SiO_2.

Conclusion

The present findings clearly demonstrate that SiO_2 and TiO_2 nanoparticles caused genotoxicity in human peripheral blood lymphocyte culture.

Acknowledgments

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