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Genotoxicity of Two Nanoparticles: Titanium Dioxide and Zinc Oxide

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Highlights

- Cytotoxic/genotoxic potential of titanium dioxide and zinc oxide nanoparticles-NPs were examined.
- Both NPs significantly increased the frequency of aberrant cells and chromosome aberration/cell.
- NPs increased sister chromatid exchange and DNA damage.
- NPs decreased mitotic index in some treatments.
- Results revealed cytotoxic and genotoxic potential of TiO_2 and ZnO NPs in human lymphocytes.

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Abstract

Nanoparticles (NPs) and nanoparticle-based materials have been increasingly used in various consumer and medical applications. However, investigations have disclosed that some NPs may generate toxic effects in different cell types and organisms. In this study, the cytotoxic and genotoxic potential of titanium dioxide (TiO_2) and zinc oxide (ZnO) NPs were examined by using four genotoxicity tests, chromosome aberrations-CAs, sister chromatid exchange-SCE, micronucleus-MN, and comet, in human lymphocytes *in vitro*. The results showed that both NPs significantly increased the frequency of aberrant cells, CA/Cell, SCE, and DNA damage, and decreased mitotic index in some treatments. These results demonstrated that TiO_2 and ZnO NPs induce genotoxic effects. Therefore, more detailed *in vitro* and *in vivo* experiments should be conducted for the safe usage of both NPs.

1. INTRODUCTION

Nanotechnology, a fast-growing and developing industry, has assisted to an increment in the production and use of engineered nanomaterials (NMs)-nanoparticles (NPs) that have at least one dimension in a range between 1 and 100 nm. NPs acquire novel and enhanced mechanical, electronic, semiconducting, optical, piezoelectric, and ultraviolet (UV)-shielding properties depend on size, shape, chemical composition, and surface chemistry compared to their bulk forms [1-2]. These new characteristics make them very important and useful materials in the World to deal with the majority of mankind's needs and problems. Metal oxide NPs such as TiO_2 and ZnO are used in various products and applications such as textiles, dyes, finishes, household products, personal care products, sports items, pharmaceuticals, prescription drugs, drug delivery, medical devices, foods, dietary supplements, and electronics. They are also applied in agriculture, environmental protection, biosensors, electronic gadgets and catalysts, plastic, rubber, cement, and coatings [2-5].

Increasing the production and usage of NPs causes elevation of human exposure and environmental contamination. Therefore, nanoparticles may interact with biological systems and can lead to unpredictable outcomes. For this reason, the toxic and especially genotoxic (DNA and chromosome damaging) potential of NPs are investigated applying various *in vitro* and *in vivo* tests. Understanding genotoxicity is a very important issue because damages in DNA and chromosomes frequently pioneer to carcinogenesis. Accumulation of this kind of damages in somatic cells may induce degenerative diseases such as

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Parkinson's and Alzheimer's. Increasing of DNA and chromosome damages in germ cells correlated with defects or heritable abnormalities in consecutive generations [1-5]. Due to all these reasons, NPs are being widely examined for their toxic effects. Some of them carried out both *in vitro* and *in vivo* have revealed toxic potentials of TiO_2 and ZnO NPs such as the breakdown of the membrane lipids, constriction of nuclear membranes, production of oxidative stress, impair in DNA, aberrations in chromosomes, gene mutations, apoptosis, lung inflammation, and consequently cancer [6-14]. On the contrary, some other investigations have shown that TiO_2 and ZnO NPs did not induce a genotoxic effect in different organisms, primary cells, or cell lines [14-17]. On the one hand, there is an increasing number of nanoparticles in human life, but on the other hand, there are many controversies regarding the genotoxicity of NPs. Therefore, efficient, cost-effective, and detailed *in vitro* studies are necessary for the evaluation of potential effects of NPs. Due to all these reasons, this study aimed to examine the genotoxic and cytotoxic effects of TiO_2 and ZnO NPs using four assays-chromosome aberrations, sister chromatid exchange, micronucleus, and comet- in human lymphocytes *in vitro*. Although there are studies on the genotoxic effects of these NPs, this is the first examination of TiO_2 and ZnO NPs using these four different and short-term genotoxicity tests.

2. MATERIAL AND METHOD

2.1. Test Materials and Chemicals

Both TiO_2 NPs [(nanopowder, <100 nm (BET), ≥97% trace metal basis, surface area 14 m^2/g, 1% Mn-doped, CAS-No: 13463-67-7)] and ZnO NPs (nanopowder, <100 nm, surface area 15-25 m^2/g, CAS-No: 1314-13-2) were bought from Sigma-Aldrich. The other chemicals used are Bromodeoxyuridine, Colchicine, Mitomycin-C, Cytochalasin-B (Sigma); EDTA, Tris, Triton X-100, DMSO, Ethidium Bromide (EtBr), Low Melting Agarose, Normal Melting Agarose, KCl (AppliChem), Trypan Blue, Biocoll, PBS, and Chromosome Medium B (Biochrome).

2.2. Preparation and Characterization of TiO_2 and ZnO Nanoparticles

Stock dispersions of TiO_2 and ZnO NPs having 100 μg/mL and 30 μg/mL concentrations, respectively, were prepared in distilled water with the aid of ultrasonic vibration for 30 minutes (J.P.Selecta; 220 V, 50-60 Hz). Stock dispersions were diluted to final concentrations and then sonicated for another 15 min. just before exposure to lymphocytes for CAs, SCEs, MN, and comet assays. 20, 40, 60, 80, and 100 μg/mL concentrations were selected for TiO_2 NPs according to the literature surveys. The same concentrations were applied for ZnO exposure. However, 1, 5, 10, 20, and 30 μg/mL concentrations were selected since the other concentrations were determined as toxic and no metaphase cell was observed.

The morphological properties of TiO_2 (100 μg/mL) and ZnO NPs (30 μg/mL) were characterized using Scanning Electron Microscope (SEM) (JEOL-JSM-6060). The hydrodynamic diameter (HD) and zeta (ζ) potentials of both NPs in the aqueous medium were measured using photon correlation spectroscopy (PCS) and the laser Doppler electrophoresis technique by using phase analysis light scattering, respectively, at 25°C using a Malvern Zeta-Sizer Nano-ZS.

2.3. Chromosome Aberration, Sister Chromatid Exchange, Micronucleus, and Comet Assays

Peripheral blood specimens were obtained from three nonsmoking healthy female donors (25-27 years old) who signed the informed consent form. This study was approved by the Ethics Committee of the Faculty of Medicine, Gazi University (03. 21.2012, No: 132), and was under the Declaration of Helsinki.

Whole blood samples were added to chromosome medium B and incubated for 72 h at 37°C. Five concentrations of TiO_2 NPs (20, 40, 60, 80, and 100 μg/mL) and ZnO NPs (1, 5, 10, 20, and 30 μg/mL) were employed to cultured cells for 24 and 48 h in CA and SCE tests, and 48 h in MN test. 2 h before harvesting, colchicine (0.06 μg/mL) was supplemented to each culture. Besides, a negative and positive control, distilled water and mitomycin-C (MMC, 0.20 μg/mL), respectively, were also run.
Evans [18] and Perry & Wolff's [19] methods were used for CA and SCE tests, respectively, with minor alterations [20, 21]. The frequency of abnormal cells and CAs/Cells were determined from 300 metaphases

in total for each treatment (100 metaphases/donor). To identify SCEs, cell cultures were supplemented with 10 µg/mL bromodeoxyuridine at the beginning of cell culture. The SCEs were determined from 75-second mitosis (25 metaphases/donor) for each treatment. The mitotic index was inspected from 3000 cells in total (1000 cells/donor). Besides, the replication index (RI) was detected from 300 cells using Equation (1):

$$RI = [M1 + 2(M2) + 3(M3)]/N \quad (1)$$

where N is the total number of metaphase, M1, M2, and M3 represent the first, second, and third metaphase, respectively [21].

For the MN assay, the same concentrations of TiO_2 and ZnO NPs were used in cell culture for the last 48 h. Cytochalasin-B (5.2 µg/mL) was supplemented at 44 h of the culture. Slides were made as described by Zengin et al. [21]. 3000 binucleated lymphocytes (1000 cells/donor) were examined for the frequency of MN for each treatment. 1500 cells (500 cells/donor) were evaluated to determine the nuclear division index using Equation (2):

$$NDI = [1xN1] + 2x[\mathrm{N2}] + 3x[N3 + N4]/n \quad (2)$$

where N1-N4 reveals the number of cells with 1-4 nuclei and "n" is the total number of cells examined [22].

Giemsa (5%, pH 6.8) was used for the staining of all the slides prepared for CA, SCE, and MN tests. Light microscope (Leica-DMLB2) at a magnification of 1000X and Leica-DFC-320 CCD camera was used to examine and capture cell images.

Using Biocoll separating solution, lymphocytes were isolated from whole blood for the comet assay and exposed to the same concentrations of both NPs used in the other assays for 30 minutes at 37ºC. Negative control and positive control (100 mM H_2O_2, 0.30 µg/mL) were also included. Cell viability was determined using the trypan blue exclusion test. The method of Singh et al. [23] was applied for comet assay with some modifications [21]. DNA damage was assessed by tail intensity (%), tail length (µm), and tail moment from 300 cells for each treatment (100 cells/donor) by using Comet Assay-IV (Perceptive Instruments Ltd.-UK) under a fluorescent attached microscope (Olympus BX-51) in a magnitude of 400x.

In CA and MN tests, treated cultures were compared with control groups using the z-test. The t-test was applied for the same assessment in SCE and comet assays. Dose-response correlations were found using the regression and correlation coefficients.

3. RESULTS AND DISCUSSION

In this research, the genotoxic potential of TiO_2 and ZnO NPs, in human lymphocytes *in vitro,* was evaluated using four different tests; CA, SCE, MN, and comet. Blood lymphocytes are critical for genotoxicity assays because pharmacologic and diagnostic agents are delivered via circulation [24]. Genotoxicity is the efficiency of physical or chemical agents to damage the genetic material, via direct or indirect ways, of the cell, and may lead to mutations and, possibly, various types of cancer [25]. Therefore, investigating the genotoxicity of NPs by using different tests is important for the safety of human life.

The shape, size, size distribution, agglomeration behavior, hydrodynamic diameter, and ζ-potential are some of the critical elements responsible for the properties of nanoparticles. These characteristics are also important for their mechanism of cellular interaction and toxicity [8, 25, 26]. In this study, SEM analysis showed that NPs were spherical and polydisperse with a particle diameter between 10 and 360 nm for TiO_2 and, between 10 and 320 nm for ZnO NPs (Figure 1). These diameters were determined to be different from the manufacturer's values. In addition to single particles, agglomerates up to 6-7 µm were also determined. Similar results were also reported in the literature [13, 27]. The mean HD was 804±15 nm for TiO_2 and 495±51 nm for ZnO NPs. However, the size reported by their commercial dealer and that determined by SEM were different from these values. These differences have resulted from various size detection methods used (e.g. BET, SEM, DLS). The size observed from DLS for both NPs was greater than the size determined

by SEM. This can be ascribed to the that DLS determines Brownian motion and consecutive size dispersion of an ensemble collection of particles in suspension and reveals mean HD which is generally larger than the diameter collected from BET or SEM analysis as it comprised a few solvent layers [28]. The ζ-potential of TiO_2 and ZnO NPs was -24.2±0.5 mV and -27.7±1.5 mV at pH 7.5, respectively. These data revealed that the surfaces of both NPs were negatively charged and located in the colloidally unstable region relatively (-30 mV≥ζ≥+30 mV) having the potential to show slight agglomeration formation confirmed by SEM [29]. Positive or negative surface charge was reported for different kinds of NPs in different suspensions used in different test systems [25, 30].

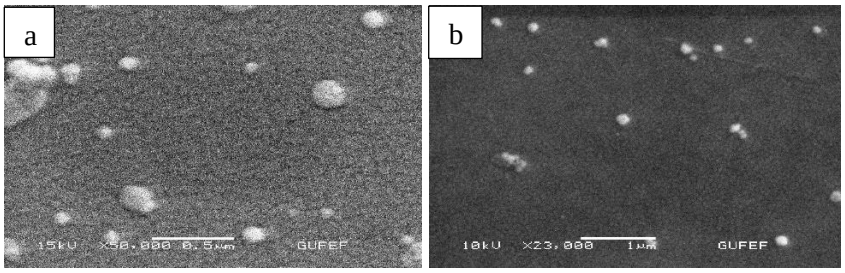

Figure 1. *SEM images of a) TiO_2 NPs, b) ZnO NPs*

In this research, TiO_2 and ZnO NPs induced various types of structural and numerical chromosome aberrations in lymphocytes (Table 1). Chromatid breaks were the most type of aberration induced by both NPs. Chromosome breaks were also generated by both NPs. Besides, while TiO NPs formed sister chromatid union, fragment, and dicentric chromosome, ZnO NPs induced chromatid exchange but not the previous three. On the other hand, while ZnO NPs induced polyploidy and endoreduplication (numerical aberrations), TiO_2 NPs induced only polyploidy. At 24 h treatment, all the concentrations of TiO_2 NPs significantly elevated the frequency of aberrant cells and CA/Cell as compared to the negative control. At 48 h application, while all the concentrations increased the same parameters, the frequency of aberrant cells was significant at 20 and 100 mg/mL, CAs /Cell was significant at 20, 80, and 100 mg/mL compared with the negative control. ZnO NPs also increased the frequency of aberrant cells (r=0.88 and r=0.69, at 24 h and 48 h treatment, respectively) and CA/Cell (r=0.98 and r=0.96 at 24 h and 48 h exposure, respectively) at both treatment periods in a dose-dependent manner. While all these increments were significant at 48 h, they were significant only at 20 and 30 mg/mL at 24 h treatment.

In the sister chromatid exchange test, TiO_2 NPs significantly elevated the amount of SCE/Cell in lymphocytes at both treatment periods. ZnO NPs, on the other hand, significantly increased SCE/Cell at higher concentrations at both 24 h (10, 20, and 30 mg/mL) and 48 h (20 and 30 mg/mL) exposures. On the contrary, both NPs decreased the mitotic index compared to the negative control. The mitotic index declined in all the concentrations of TiO NPs at 24 h treatments. However, only two of them (60 and 80 mg/mL) were statistically significant as compared to the negative control value. At 48 h exposure, there was no statistical difference between treated and negative control groups. In ZnO NPs treated lymphocytes, MI decreased at all the concentrations, among them 5, 10, and 30 mg/mL at 24 h, and only 30 mg/mL was significant compared to the negative controls (Table 2).

TiO_2 NPs elevated the formation of micronucleus at all the concentrations (except 100 μg/mL) as compared to the negative control value, however, this elevation was not significant. Generally, 1 micronucleus was observed in BNCs, however, 2 and 3 MNi were also observed. ZnO NPs also increased MN% in human lymphocytes. However, this increase was significant at only two highest (20 ve 30 μg/mL) concentrations. ZnO NPs induced generally 1MN, however, 2 MNi were also observed in some lymphocytes. The value of the nuclear division index (NDI) was not altered by TiO_2 and ZnO NPs (Table 3). In this research, the comet assay was also used (Table 4). Cell viability was higher than 95% in human lymphocytes after NP exposure. In TiO_2 exposure, tail intensity (r=0.77), tail length (r=0.71), and tail moment (r=0.86) showed dose-dependent increase, in general. However, only 100 μg/mL concentration induced significant tail intensity as compared to the negative control. In ZnO treatment, comet tail length increased at all the concentrations (except 30 μg/mL, decreased non-significantly) as compared to the control. However, the increment was significant at only 5 and 20 μg/mL. In contrast, tail intensity decreased at all the concentrations but

increased at 10 μg/mL. However, decreasing was significant only at 30 μg/mL. The tail moment showed both increasing and decreasing, however, none of them was significant. Regression analyses showed that there is a weak correlation between increasing concentrations of ZnO NPs and DNA damage (r=0.61, 0.63, and 0.63 for tail intensity, tail length, and tail moment, respectively).

Table 1. Effects of TiO_2 and ZnO NPs on chromosome aberrations

Test substance	Treatment		Aberrations								Abnormal cell ± SE (%)	CA/Cell ± SE
			Structural						Numerical			
	Period (h)	Concent. (μg/mL)	ctb	csb	cte	f	scu	dis	p	e		
N. Control	24	0	2	-	-	-	2	-	-	-	1.33±0.66	0.013±0.01
MMC	24	0.2	32	20	5	7	3	-	-	-	20.33±2.32	0.223±0.02
TiO_2 NP	24	20	17	3	-	2	-	-	-	-	6.00±1.37**	0.073±0.02***
		40	14	3	-	-	4	-	1	-	6.67±1.44***	0.070±0.02***
		60	11	3	-	-	1	-	-	-	4.33±1.17*	0.050±0.02**
		80	8	6	-	-	-	1	-	-	5.00±1.26*	0.050±0.02**
		100	11	1	-	-	2	-	-	-	4.67±1.22*	0.047±0.02*
N. Control		0	3	1	-	-	-	-	-	-	1.33±0.66	0.013±0.01
MMC	48	0.2	16	26	4	-	5	1	1	-	14.00±2.00	0.173±0.02
TiO_2 NP	48	20	10	1	-	2	2	-	1	-	5.33±1.30**	0.047±0.01*
	48	40	7	-	-	-	1	1	-	-	3.00±0.98	0.030±0.01
		60	5	1	-	-	2	1	1	-	3.33±1.03	0.027±0.01
		80	5	1	-	-	5	-	1	-	3.33±1.03	0.037±0.01*
		100	6	-	-	-	5	-	1	-	4.00±1.13*	0.037±0.01*
Total Number			94	19	-	4	22	3	5			
N. Control	24	0	5	1	-	-			-	-	1.67±1.26	0.020±0.008
MMC	24	0.2	19	10	9	5			-	-	13.67±1.98	0.143±0.022
ZnO NP	24	1	11	3	-	-			2	-	4.33±1.18	0.047±0.012
		5	5	3	-	5			-	-	4.00±1.13	0.043±0.012
		10	10	-	-	2			2	1	3.67±1.08	0.040±0.011
		20	14	3	1	1			2	3	5.33±1.30*	0.063±0.014**
		30	16	8	3	4			3	3	9.00±1.65***	0.103±0.017***
N. Control	48	0	3	-	-	-			-	-	1.00±0.57	0.010±0.006
MMC	48	0.2	39	26	13	11			-	-	24.33±2.48	0.297±0.209
ZnO NP	48	1	11	3	-	1			3	-	5.00±1.26**	0.050±0.012**
		5	15	1	-	-			1	-	5.33±1.30**	0.053±0.013**
		10	5	2	-	4			2	-	3.67±1.08*	0.037±0.011*
		20	7	4	-	2			1	-	4.33±1.18*	0.043±0.012*
		30	14	4	-	2			1	1	6.67±1.44***	0.067±0.014***
Total number			108	31	4	20			17	8		

Ctd: chromatid break, csb: chromosome break, cte: chromatid exchange, f: fragment, scu: sister chromatid union, dic: dicentric chromosome, p: polyploidy, e: endoreduplication, N. Control: negative control, MMC: positive control (Mitomycin-C), SE: standard error.

*P<0.05 versus negative control

**P<0.01 versus negative control

***P<0.001 versus negative control (z-test)

CA and SCE tests are important because elevations in these aberrations in peripheral lymphocytes (PLs) have significantly increased the risk of cancer progression [31]. Similarly, an association has been determined between the generation of MN in the PLs of healthy individuals and subsequent risk of cancer in a large range of experimental studies [32]. Comet assay was suggested as the reliable indicators of DNA damage [33, 34]. Therefore, all these tests are very sensitive for the determination of genotoxicity and biosafety of NPs [25].

Table 2. Effects of TiO_2 and ZnO NPs on sister chromatid exchange, replicative index, and mitotic index

Test substance	Treatment		Min.-max. SCE	SCE/Cell ± SE	M_1	M_2	M_3	RI ± SE	MI ± SE (%)
	Period (h)	Concent. (μg/mL)							
N. Cont.	24	0	0-12	3.80±0.26	46	85	169	2.41±0.04	5.57±0.42
MMC	24	0.2	10-55	25.45±1.06	51	124	125	2.25±0.04	4.50±0.38
TiO_2 NP	24	20	2-12	5.28±0.29*	45	83	172	2.42±0.04	4.57±0.38
		40	0-11	5.12±0.29*	61	84	155	2.31±0.04	5.20±0.40
		60	1-19	6.16±0.35*	55	91	154	2.33±0.03	4.43±0.38*
		80	0-13	4.95±0.27*	83	104	113	2.10±0.05	4.43±0.38*
		100	1-14	5.57±0.32*	71	88	141	2.23±0.05	4.60±0.38
N. Cont.	48	0	0-9	3.51±0.29	81	107	112	2.10±0.05	4.93±0.39
MMC	48	0,2	12-48	37.81±1.62	131	148	21	1.60±0.04	3.27±0.32
TiO_2 NP	48	20	2-14	5.55±0.27*	66	110	124	2.19±0.04	4.37±0.37
		40	0-12	5.03±0.29*	65	90	145	2,.7±0.05	4.97±0.40
		60	1-13	5.95±0.28*	52	79	169	2.39±0.04	4.97±0.40
		80	1-11	5.51±0.27*	59	92	149	2.30±0.05	4.87±0.39
		100	0-12	5.60±0.28*	39	81	180	2.47±0.04	5.33±0.41
N. Cont.	24	0	1-9	4.28±0.19	43	82	175	2.44±0.04	6.43±0.45
MMC	24	0,2	10-30	22.49±0.79	71	131	98	2.09±0.04	4.13±0.36
ZnO NP	24	1	1-10	4.56±0.23	61	84	155	2.31±0.04	5.40±0.41
		5	1-9	4.44±0.20	60	87	153	2.31±0.04	5.06±0.40*
		10	1-10	5.08±0.24*	58	97	145	2.29±0.04	5.06±0.40*
		20	2-12	5.51±0.26*	73	109	118	2.15±0.05	5.33±0.41
		30	1-9	4.99±0.21*	133	88	79	1.82±0.05	3.53±0.34**
N. Cont.	48	0	1-8	4.33±0.23	72	78	150	2.26±0.05	6.67±0.46
MMC	48	0,2	14-47	32.32±1.13	152	106	42	1.63±0,04	3.33±0.33
ZnO NP	48	1	2-10	4.72±0.19	60	82	158	2.33±0.05	5.73±0.42
		5	1-9	4.81±0.23	42	101	157	2.38±0.04	5.57±0.42
		10	0-9	4.77±0.24	50	100	150	2.33±0.05	6.00±0.43
		20	1-11	5.09±0.25*	87	98	115	2.09±0.05	5.67±0.42
		30	3-13	5.21±0.22*	119	82	99	1.93±0.05	3.77±0.35**

*P<0.05 versus negative control

***P<0.001 versus negative control (t-test)

Table 3. Effects of TiO_2 and ZnO NPs on micronucleus and nuclear division index

Test substance	Treatment		MN ± SE (%)	Nuclear division index ± SE
	Period (h)	Concent. (μg/mL)		
N. Cont.	48	0	0.13±0.066	1.57±0.32
MMC	48	0.2	2.57±0.289	1.52±0.31
TiO_2 NP	48	20	0.30±0.099	1.66±0.33
		40	0.30±0.099	1.60±0.32
		60	0.30±0.099	1.62±0.33
		80	0.17±0.075	1.61±0.33
		100	0.13±0.066	1.60±0.32
N. Cont.	48	0	0.13±0.066	2.00±0.36
MMC	48	0.2	2.37±0.278	1.86±0.35
ZnO NP	48	1	0.23±0.087	1.94±0.36
		5	0.27±0.095	1.99±0.36
		10	0.33±0.105	1.98±0.36
		20	0.40±0.115*	1.98±0.36
		30	0.40±0.115*	1.94±0.36

*P<0.05 versus negative control

Table 4. Effects of TiO_2 and ZnO NPs on DNA damage

Test Substance	Treatment		Tail intensity (%)	Tail Length (μm)	Tail Moment
	Period (min)	Concent. (μg/mL)			
N. Control	30	0	4,95±0,44	52,70±0,55	1,03±0,09
H_2O_2 (μM)	30	100	5,75±0,50	67,36±1,75	1,46±0,13
TiO_2 NP	30	20	4,71±0,53	51,60±0,64	1,01±0,11
		40	6,04±0,70	53,49±0,68	1,59±0,29
		60	6,19±0,70	54,29±0,70	1,73±0,36
		80	6,18±0,65	54,38±0,63	1,49±0,25
		100	6,12±0,75	57,59±1,02*	1,90±0,41
N. Control	30	0	14,39±1,02	55,84±0,64	3,75±0,38
H_2O_2 (μM)	30	100	17,32±1,36	95,85±3,22	6,83±0,77
ZnO NP	30	1	11,98±0,82	57,82±0,88	3,06±0,28
		5	12,33±0,93	59,04±1,10*	3,15±0,34
		10	15,26±1,00	56,87±0,80	3,80±0,37
		20	12,58±1,04	60,70±0,96*	3,81±0,51
		30	11,20±0,81*	54,24±0,75	2,74±0,27

*P<0.05 versus negative control (t-test)

Our results are agreed with the results of some of the previous investigations for the cytotoxicity, genotoxicity, and DNA damaging effects of both TiO_2 and ZnO NPs. However, negative results were also observed for these NPs. For example, Patel et al. reported a dose-related rise in the frequency of CAs and DNA damage after 24 h of exposure of human lymphocytes with 25 (did not induce CAs), 75, and 125 μM TiO_2 NPs [25]. Exposure of murine RAW 264.7 cells (10, 25, 50, 75, and 100 mg/mL) and Albino mice (200 and 500 mg/kg) to TiO_2-NPs decreased cell viability in a concentration-dependent manner and induced significant dose-dependent DNA damage using comet assay (only 500 mg/kg in mice). In *in vivo* MN assay, carried out in Albino mice, while 500 mg/kg of TiO_2 NPs significantly increased micronucleated polychromatic erythrocytes (MNPCE%), 200 mg/kg TiO_2 NPs did not alter MNPCE%. Similarly, CAs significantly increased in mice exposed to 500 mg/kg of TiO_2 NPs [11]. TiO_2 NPs induced a positive and significant induction of CAs at 24h treatment at 6.25 μg/mL. However, 12.5, 25, 50, 100, 150, and 300 μg/mL concentrations did not induce significant aberrations. After 48 h exposure, 100 and 300 μg/mL induced a significant increment in total aberrations. It was reported that longer-term exposure may facilitate the entrance of the smallest internalized nanoparticles to the nucleus in the course of cell division and allows direct contact with DNA or chromosomes and thus produce CA [35]. Ghosh et al. [13] examined toxic effects of the same ZnO NPs we investigated and observed that 3 h treatment of 20, 40, 80, and 100 μg/mL of NPs were not cytotoxic in peripheral human blood mononuclear cells (PBMCs) *in vitro.* However, they induced a weak genotoxic effect and a significant reduction in mitochondrial membrane potential (MMP), generated reactive oxygen species (ROS), and lead to apoptosis. In bone marrow cells in Swiss albino mice, chromosome aberrations and micronuclei were observed at 25, 50, and 100 mg/kg body weight. Besides, decreasing in MMP and increasing oxidative stress was evident. Formation of DNA disturbance and oxidative stress with a coincident reduction in antioxidant enzymes were also determined in liver cells. Although ZnO NPs we used are the same as the ones Gosh et al.'s used in their study, our NPs were ~100 nm in size and ranged from 10 to 320 nm by DLS, theirs were ~80 nm and ranged from 282 to 345 nm by DLS. Treatment concentrations and periods were also different from ours. Therefore, some differences in the results were observed between these two studies. Fadoju et al. [12] reported a significant increment in CAs and reduction in the mitotic index in *Allium cepa* after exposure to TiO_2 and ZnO NPs and their 1:1 combination.

Low or no cytotoxicity was observed by WST-1 test using trademark TiO_2-NPs. While P25 and food-grade TiO_2 NPs, and engineered platelet TiO_2 NPs induced significant DNA damage, genotoxicity was not observed with the bipyramid and rod TiO_2 NPs using comet assay [36]. Only 100 μg/mL of commercial P25 NPs induced cytotoxicity after 24 h treatment [37]. While food-grade TiO_2 NPs caused cytotoxicity on Caco-2 cells, they did induce any cytotoxic effect on HCT116 up to 100 μg/cm^2 [38]. Although a significant number of MN was determined at 20 μg/mL of TiO_2 NPs in HepG2 cells, further treatment with higher

concentrations showed a decrease which may be due to the accumulation of NPs [30]. Different kinds of ZnO NPs did not generate genotoxicity at any dose *in vivo* in mice [17, 27].

Genotoxicity of NPs can be classified in two different ways; primary and secondary. The primary genotoxicity exists in two types: either direct or indirect mechanisms. Direct primary genotoxicity occurs after the NPs penetrate the nucleus, interact with genetic material, and inhibit DNA replication and/or transcription. Besides, NPs may interact with chromosomes during mitosis and induce chromosome breakage or chromosome loss which are called the clastogenic and aneugenic effects, respectively. Indirect primary genotoxicity is induced due to the NP-mediated ROS generation, toxic ions released from NPs, the interaction of NPs with nuclear proteins important for DNA replication, transcription or repair, prevention of antioxidant defense, and disruption of cell cycle checkpoints. Secondary genotoxicity arises due to the production of ROS during inflammation, and its interaction with DNA [25, 26, 37].

In conclusion, in this research, TiO_2 and ZnO NPs were evaluated by using four genotoxic assays; CA, SCE, MN, and comet assays that have different mechanisms to determine genetic damage, for the first time. While both NPs induced CAs and SCEs in human lymphocytes, MN frequency and DNA damage determined by comet assay was not significantly greater than those of their controls. This difference may be explained by the fact that NPs tend to constitute bigger agglomerates and may not be able to enter the nuclei and induce breaks in DNA due to the size of the agglomerates. The other possibility is that cells at a late stage of apoptosis might be observing as cell debris and thus not being detected by the assays. It is reported that while breaks rise DNA migrations, DNA binding and crosslinks can decrease DNA migrations that can also be determined by the comet assay [39].

Toxicity results of TiO_2 and ZnO NPs reveal high fluctuations. This may have resulted from various factors such as the physicochemical features of NPs, diverse techniques used to make NP dispersions, variations in NPs size, shape, electrical charge, stability, concentrations, and treatment procedures [40]. All these results reveal that more detailed *in vitro* and *in vivo* genotoxicity studies using different methods should be conducted for the safe usage of these NPs.

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CONFLICTS OF INTEREST

No conflict of interest was declared by the authors.

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