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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 TiO2 and ZnO NPs in human lymphocytes.

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 (TiO2) 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 TiO2 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 TiO2 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 TiO₂ 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 TiO₂ 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 TiO₂ 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 TiO₂ and ZnO NPs using these four different and short-term genotoxicity tests.

2. MATERIAL AND METHOD

2.1. Test Materials and Chemicals

Both TiO₂ NPs [(nanopowder, <100 nm (BET), ≥97% trace metal basis, surface area 14 m²/g, 1% Mn-doped, CAS-No: 13463-67-7)] and ZnO NPs (nanopowder, <100 nm, surface area 15-25 m²/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 TiO₂ and ZnO Nanoparticles

Stock dispersions of TiO₂ 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 TiO₂ 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 TiO₂ (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 TiO₂ 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):

\[ RI = \frac{M_1 + 2(M_2) + 3(M_3)}{N} \]  

where \( N \) is the total number of metaphase, \( M_1 \), \( M_2 \), and \( M_3 \) 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):

\[ NDI = \frac{[1\times N_1] + 2\times [N_2] + 3\times [N_3 + N_4]}{n} \]  

where \( N_1-N_4 \) 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 37°C. Negative control and positive control (100 mM H\(_2\)O\(_2\), 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 $\zeta$-potential of TiO$_2$ and ZnO NPs was $-24.2 \pm 0.5$ mV and $-27.7 \pm 1.5$ 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$\leq\zeta\leq+30$ 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, CA/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$_2$ 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/Cel ± SE |
|---------------|-----------|-------------|-------------------------|-------------|
|               | Period (h) | Concent. (µg/mL) | Abr. Ctb | Abr. Csb | Abr. Cte | Abr. F | Abr. Scu | Abr. Dis | Abr. p | Abr. e |
| N. Control    | 24        | 0            | 2    | 3    | -     | -     | 2    | -    | -    | 1.33±0.66 | 0.013±0.01 |
| MMC          | 24        | 0.2          | 20   | 7    | -     | -     | 3    | -    | -    | 20.33±2.32 | 0.023±0.02 |
| TiO$_2$ NP   | 24        | 20           | 3    | -    | 2     | -     | -    | -    | -    | 6.00±1.37** | 0.003±0.02*** |
|              | 40        | 14           | 3    | -    | 4     | -     | 1    | -    | -    | 6.67±1.43** | 0.007±0.02*** |
|              | 60        | 11           | 3    | -    | -     | 1     | -    | -    | -    | 4.33±1.17** | 0.001±0.02** |
|              | 80        | 8            | 6    | -    | -     | 1     | -    | -    | -    | 3.67±1.23*  | 0.008±0.02*  |
|              | 100       | 11           | 1    | -    | -     | 2     | -    | -    | -    | 4.67±1.22*  | 0.047±0.02*  |
| N. Control    | 0         | 3            | 1    | -    | -     | -     | -    | -    | -    | 1.33±0.66 | 0.043±0.01 |
| MMC          | 48        | 0.2          | 16   | 26   | 5     | 1     | 1    | -    | -    | 14.00±1.00 | 0.173±0.02 |
| TiO$_2$ NP   | 48        | 20           | 10   | 1    | -     | 2     | 3    | -    | -    | 3.33±1.30  | 0.047±0.01*  |
|              | 48        | 40           | 7    | -    | -     | 1     | 1    | -    | -    | 6.00±0.98  | 0.030±0.01  |
|              | 60        | 5            | 1    | -    | -     | 2     | 1    | -    | -    | 3.33±1.05  | 0.027±0.01  |
|              | 80        | 5            | 1    | -    | -     | 5     | 1    | -    | -    | 3.33±0.03  | 0.037±0.01*  |
|              | 100       | 6            | -    | -    | -     | 5     | -    | 1    | -    | 4.00±1.13*  | 0.037±0.01*  |

| Total Number | 24        | 0            | 5    | 1    | -     | -     | 1    | 7    | -    | 1.67±1.26  | 0.020±0.008 |
| N. Control    | 24        | 0.2          | 19   | 10   | 9     | 5    | -    | -    | -    | 13.67±1.98 | 0.143±0.022 |
| ZnO NP        | 24        | 1            | 11   | 3    | -     | -     | -    | -    | -    | 4.33±1.18  | 0.047±0.012 |
|              | 5         | 5            | 3    | 5    | -     | -     | -    | -    | -    | 4.00±1.13  | 0.043±0.012 |
|              | 10        | 10           | -    | -    | 6     | 2     | 1    | 3    | -    | 3.67±1.08  | 0.040±0.011 |
|              | 20        | 14           | 3    | 1    | 4     | 2     | 3    | 5.33±1.30* | 0.063±0.014*** |
|              | 30        | 16           | 8    | 4    | 7     | 3     | 3    | 9.00±1.65*** | 0.103±0.17*** |
| 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    | 1    | -    | -    | 5.33±1.30** | 0.053±0.013** |
|              | 10        | 5            | -    | -    | 4     | 2     | 2    | 3.67±1.08* | 0.037±0.011*  |
|              | 10        | 5            | -    | -    | 4     | 2     | 2    | 3.67±1.08* | 0.037±0.011*  |
|              | 20        | 7            | 4    | -    | 2     | 1    | 1    | 4.33±1.18* | 0.043±0.012*  |
|              | 20        | 7            | 4    | -    | 2     | 1    | 1    | 4.33±1.18* | 0.043±0.012*  |
|              | 30        | 14           | 4    | 5    | 1     | 1    | 1    | 6.67±1.44*** | 0.067±0.014*** |

| Total number | 104       | 31           | 20  | 17    | 8 |

Ctd: chromatid break, Csb: chromatid 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 Period (h) | Concent. (μg/mL) | Min.-max. SCE | SCE/Cell ± SE | M$_1$ | M$_2$ | M$_3$ | RI ± SE | MI ± SE (%) |
|----------------|---------------------|------------------|----------------|----------------|------|------|------|--------|-------------|
| N. Cont.       | 24 0                | 0-12             | 3.80±0.26      | 2.41±0.04      | 5.75±0.42 |
| MMC            | 24 0.2              | 10-55            | 25.45±1.06     | 2.25±0.04      | 4.50±0.38 |
| TiO$_2$ NP     | 24 0.2              | 10-55            | 5.28±0.29      | 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      | 2.10±0.05      | 4.93±0.39 |
| MMC            | 48 0.2              | 12-48            | 37.81±1.62     | 1.60±0.04      | 3.27±0.32 |
| TiO$_2$ NP     | 48 0.2              | 12-48            | 5.55±0.27      | 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      | 4.63±0.39    |
| 100 0-12       | 5.60±0.28*          | 39 81 180       | 2.47±0.04      | 5.35±0.45    |
| N. Cont.       | 48 0                | 1-9              | 4.28±0.19      | 2.44±0.04      | 6.43±0.45 |
| MMC            | 48 0.2              | 10-30            | 22.49±0.79     | 2.09±0.06      | 4.13±0.36 |
| ZnO NP         | 48 0.2              | 10-30            | 4.56±0.23      | 2.31±0.04      | 5.10±0.41 |
| 5 1-9          | 4.44±0.20           | 60 87 153       | 2.31±0.04      | 5.06±0.40*   |
| 10 1-13        | 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      | 2.16±0.05      | 5.33±0.44*   |
| 30 1-9         | 4.99±0.21*          | 133 88 206      | 1.86±0.05      | 5.40±0.45*** |
| N. Cont.       | 48 0                | 1-8              | 4.33±0.23      | 2.26±0.04      | 6.67±0.46 |
| MMC            | 48 0.2              | 14-47            | 32.32±1.13     | 1.63±0.04      | 3.33±0.33 |
| ZnO NP         | 48 0.2              | 14-47            | 4.72±0.19      | 2.33±0.05      | 5.73±0.42 |
| 5 1-9          | 4.81±0.23           | 60 101 158      | 2.33±0.05      | 5.57±0.42    |
| 10 0-9         | 4.77±0.24           | 50 100 150      | 2.21±0.05      | 6.00±0.43    |
| 20 1-11        | 5.09±0.25           | 87 98 115       | 2.3±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 Period (h) | Concent. (μg/mL) | MN±SE (%) | Nuclear division index ± SE |
|----------------|---------------------|------------------|-----------|---------------------------|
| N. Cont.       | 48 0                | 0.13±0.066       | 1.57±0.32 |
| MMC            | 48 0.2              | 2.37±0.289       | 1.52±0.31 |
| TiO$_2$ NP     | 48 0.2              | 0.30±0.099       | 1.66±0.33 |
| 40 0-12        | 0.30±0.099          | 1.60±0.32       |
| 60 0-12        | 0.30±0.099          | 1.62±0.33       |
| 80 0-12        | 0.17±0.075          | 1.61±0.33       |
| 100 0-12       | 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 0.2              | 0.23±0.087       | 1.94±0.36 |
| 10 0-9         | 0.27±0.095          | 1.99±0.36       |
| 20 0-11        | 0.33±0.105          | 1.98±0.36       |
| 30 0-11        | 0.40±0.115*         | 1.98±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$_2$O$_2$ (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$_2$O$_2$ (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 vitro MN assay, carried out in Albino mice, while 500 mg/kg of TiO$_2$ NPs significantly increased micronucleated polychromatic erythrocytes (MNPC%). 200 mg/kg TiO$_2$ NPs did not alter MNPC%. 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 24 h 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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