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ORIGINAL ARTICLE

Interaction of titanium dioxide and zinc oxide nanoparticles induced cytogenotoxicity in *Allium cepa*

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Abstract

The extensive production and utilisation of titanium dioxide (TiO_2) and zinc oxide (ZnO) nanoparticles (NPs) in consumable items may enhance significant increase in fauna and flora exposure. Studies showing the interactive effect of NPs in biological systems are limited. Herein, we showed the cytogenotoxic effects of TiO_2 and ZnO NPs, and their mixture (1:1) using the *Allium cepa* assay. Mitotic index (MI) and chromosomal aberrations (CAs) were assessed in *A. cepa* L. bulbs exposed to each NP and their mixture at concentrations of 5, 10, 20, 40 and 80 mg L^{-1}, respectively. The recovery effect of the root tip cells from the cytogenotoxic effects of the nanoparticles was also investigated. TiO_2, ZnO NPs and their mixture significantly ($p < 0.05$) induced increase in CA and reduction in MI in *A. cepa* root cells, but the mixture induced the highest frequency of CA and reduction in MI. When the treated meristematic cells were placed in water for recovery, there were reduction in the number of aberrant cells in *A. cepa* exposed to TiO_2 and the mixture. Interactive factor analysis of the effects of the mixture showed antagonism. The aberrations induced by TiO_2 NPs appeared to be transient while those induced by ZnO NPs may be transmissible due to the increase in frequency of aberrations in the recovery test. This finding showed the potential of tested NPs to induce mutation in somatic cells, and is of public and environmental health significance.

Keywords *Allium cepa* · Chromosome aberration · Titanium dioxide nanoparticle · Zinc oxide · Nanoparticle · Mitotic index

Introduction

Exponential growth in nanotechnology has led to an increase in the application of nanoparticles (NPs) in diverse areas of consumer products such as personal care products, pharmaceuticals, food, house hold products, electronic devices, sports items, and paints to enhance survival and comfort [9, 34]. Nanoparticles are particles with at least a dimensional feature between 1 and 100 nm [25], possessing excellent physical and chemical properties that make them different from their bulk counterparts [30, 35]. These physicochemical properties make them the most sought materials in the world as they are used in disease diagnosis and treatment, remediation of contaminated air and water, and in solving majority of mankind's problems [53].

Nanoparticles can either be natural or engineered [55]. Engineered NPs which include metal oxides, metals, quantum dots, carbon nanotubes, dendrimers and fullerenes [44] are highly sought for in industrial applications and consumer products due to their fantastic physicochemical properties [53]. An increase in the production of consumer products with NPs may increase human exposure and environmental contamination [35]. Humans and other organisms in the environment including plants and aquatic organisms, are also at the risk of exposure to these NPs as they are deliberately or accidentally released into the

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environment via the products containing them. Nanoparticles are capable of interacting with a variety of biological materials ranging from cells to organ systems because of their small size and large surface area to volume ratio [30]. They can penetrate the tissue, blood–brain and testis barriers, alter the integrity of the plasma membrane and induce genetic damage via different but yet unclear mechanisms [42, 45].

Titanium dioxide (TiO_2) and zinc oxide (ZnO) are examples of metal oxide NPs that have greatly received attention in the pharmaceutical, industrial and agricultural sectors because of the antimicrobial, photocatalytic and anticorrosive properties [50]. More than 1.2 million and 4.3 million tonnes of ZnO and TiO_2 NPs, respectively, are produced yearly [36]. Due to the widespread use, TiO_2 and ZnO can enter aquatic and terrestrial environment and potentially affect the indigenous organisms. Reports on accumulation in the environment showed that TiO_2 NP accumulate in sludge-treated soils followed by sediments and landfills (approx. 8400 t/a and 7600 t/a and 7000 t/a), while ZnO NP accumulate in sediments (1300 t/a), in natural and urban soil (300 t/a), as well as at landfills (200 t/a) [8]. The two NPs were also reported to co-exist in the natural water of China [15]. This provides possibility of co-existence in other clime where both NPs are also in use.

Studies on mammalian exposure to TiO_2 and ZnO NPs are numerous in the literature, however, studies on plant's exposure are limited despite the fact that plants are a major part of the ecosystem and are the recipients of numerous environmental contaminants [16, 22]. The fate and transport of metal oxide NPs in the environment through the plant system is very critical. The cytotoxic and genotoxic effects of TiO_2 and ZnO NPs have been investigated in a few plant systems including *Arabidopsis thaliana* [38], *Lolium perenne* [41], *Vicia faba* [20, 21], *Allium sativum* [51], *Fagopyrum esculentum* [39], *Triticum aestivum* [49] and *Nicotiana tabacum* [20].

Few studies have reported the potential DNA damaging effect of TiO_2 or ZnO NPs in *Allium cepa* meristematic cell [16, 31, 36, 47]. However, the potential genotoxic effect of mixture of TiO_2 and ZnO NPs has not been investigated using the *A. cepa* assay. Evaluation of cytogenotoxic effect of mixture of TiO_2 and ZnO is important because both constitute the two most widely used engineered nanomaterials [15]. Their co-existence was reported to effectively enhance their stability [15], hence potential for a stable interaction with the bio system.

The *A. cepa* assay has been used as a standard model in assessing the toxic and genotoxic effects of several environmental contaminants and pollutants such as industrial effluents [4, 13], pesticides [33], e-waste leachates [5, 6]; and medicinal plant extracts [3, 46]. This is because it has a short preparation time; it is cheap; it correlates well with mammalian assays; it is very sensitive by producing several genetic and chromosomal anomalies; and it has high dividing cells with low amount of monocentric chromosomes [40]. Hence, this study was designed to investigate the individual and interactive cytogenotoxic potential of TiO_2 and ZnO NPs using the *A. cepa* assay.

Materials and methods

Nanoparticles

TiO_2 (anatase, CAS No: 1317-70-0) and ZnO (CAS No: 1314-13-2) nanopowders were obtained from Sigma Aldrich, USA. The physical characteristics of TiO_2 NPs according to the manufacturer's data are size: < 25 nm, purity: 99.7% anatase, and molecular weight of 79.87 g/mol; while ZnO NPs has size of < 100 nm, purity: 99.5%, surface area of 15–25 $m^2 g^{-1}$ and molecular weight of 81.39 g/mol.

Nanoparticles preparation and physicochemical characterisation

Detailed physicochemical characterisations of TiO_2 NPs, ZnO NPs and their mixture (1:1) have been published in our previous study [14]. The molar ratio of the mixture of TiO_2 and ZnO NPs was systematically chosen due to the study of Jiang et al. [26], which showed that 1:1 molar ratio of TiO_2 and ZnO NPs exhibited the highest photocatalytic activity for the decolourization of C.I. Basic Blue 41. Both NPs and their mixture (1:1) were suspended in distilled water and dispersed for 10 min using an ultrasonicator (Bandelin, Sonorex digitec, Germany) and further vortexed for 5 min to prevent agglomeration of the nanoparticles. Five concentrations of each NPs or mixture corresponding to 5, 10, 20, 40 and 80 mg L^{-1} were prepared. These concentrations were chosen based on previous reports which implied an IC_{50} of less than 50 mg L^{-1} for mitotic index (MI) by each of the tested NPs [47, 54]. Hence, we utilised four concentrations below 50 mg L^{-1} and one above it. This allowed observations of nuclear anomalies when the cell cycle is not inhibited and when it is significantly inhibited.

Allium cepa assay

Onions (*Allium cepa*, L., 2n = 16, Family *Amaryllidaceae*) obtained commercially in Ibadan, Nigeria, were sun-dried for 2 weeks and used according to standard [17] to evaluate the potential genotoxic and recovery effects of the root tip cells. Four onion bulbs were utilised per concentration of each of the tested NPs, with distilled water as negative control. For the genotoxicity test, four bulbs per concentration were placed on beakers containing distilled water for 48 h

and then transferred to the respective treatment and left for 24 h. At 24 h, the root tips of the onions were harvested and the meristematic region from the cut root tips was processed for slide preparation.

In a separate experiment investigating recovery effect, another set of four bulbs per concentration were treated as in the genotoxicity test, but transferred into distilled water for another 24 h. The meristematic region from the root tips of these bulbs was thereafter processed for slide preparation.

Cytogenetic analysis

Slides were prepared following the squash protocols [17]. The cut root tips from each concentration were separately fixed in methanol:glacial acetic acid (3:1 v/v), after which the roots were hydrolyzed in 1 N HCl at 60 °C for 5 min and then washed three times in distilled water. Two root tips were squashed on each slide and stained with acetocarmine for 10 min. Six slides were prepared per concentration, out of which four (at 1000 cells per slide) were randomly observed at × 1000. Chromosomal aberrations were characterised and classified. The MI was calculated as the number of dividing cells per 1000 observed cells [17] at each concentration. The frequency of aberrant cells (%) was calculated based on the number of aberrant cells per total cells scored per slide for each concentration of the NPs and their mixture.

Interaction factor (IF)

The interaction factor for TiO_2 and ZnO NPs for the CAs was calculated according to Katsifis et al. [27]:

$$IF = M - T - Z + NC$$

$$SD_{IF} = \sqrt{(SD_M)^2 + (SD_T)^2 + (SD_Z)^2 + (SD_{NC})^2}$$

where M is frequency of CA for the mixture, T is the frequency of CA for TiO_2 NPs, Z is the frequency of CA for ZnO NPs and NC is the frequency of CA for the negative control (distilled water). SD_{IF} is the standard deviation of the interaction factor, SD_M is the standard deviation of the mixture, SD_T is the standard deviation of TiO_2 NPs, SD_Z is the standard deviation of ZnO NPs, and SD_{NC} is the standard deviation of the negative control. A negative IF value represents antagonism, a positive IF value represents synergism while a zero IF value represents additivity.

Statistical analysis

Statistical analysis was performed using the SPSS 20.0® software package. The frequency of CAs per total cells scored was analysed and presented as Mean ± SD. Data on MI and CAs were analysed using one way ANOVA followed by Duncan test ($p < 0.05$).

Results

The cytological effects of TiO_2 and ZnO NPs and their mixture (1:1) are presented in Table 1. The data showed a concentration-dependent decrease in MI in TiO_2 NPs exposed *A. cepa* root cells compared to the negative control. Similarly, a decrease in MI was observed in *A. cepa* root cells exposed to ZnO and TiO_2/ZnO NPs mixture compared to the negative control except at the 10 mg L^{-1} of the mixture. In the recovery study, decrease in MI compared to the negative control was also observed at all the tested concentrations of TiO_2, ZnO (except at the 5 mg L^{-1} of TiO_2 NPs) and the mixture. The MI in the recovery groups was higher than in the genotoxicity groups for some concentrations of ZnO NPs and mixture-treated onions (Table 1).

Different types of CAs such as anaphase bridge, c-mitosis, disturbed spindle, lagging chromosome, sticky and fragmented chromosomes (Fig. 1a–l) were induced by the NPs and their mixture in *A. cepa* root tip cells. All the tested concentrations of TiO_2, ZnO and the mixture induced significant CAs ($p < 0.05$) compared to the negative control except at 10 and 80 mg L^{-1} for TiO_2 and 5 mg L^{-1} for both ZnO and the mixture. The highest frequency of CAs in cells exposed to TiO_2 NPs was observed at 20 mg L^{-1} after which a decline in frequency of CAs was observed. Compared to the genotoxicity group, a reduction in frequency of aberration was observed in the recovery group of TiO_2 NPs at the tested concentrations. An increase in frequency of CAs was observed in the recovery group of ZnO NPs compared to the genotoxic group at the tested concentrations except at 10 mg L^{-1}. An increase in the frequency of CAs was observed in the recovery group of the mixture compared to those in the genotoxic group except at the 20 and 40 mg L^{-1}. Interaction factor analysis showed that the NPs acted antagonistically in the mixture.

Discussion

The *A. cepa* CA assay is a commonly used assay for the screening of mutagens. It is useful for evaluating and ranking environmental chemicals with reference to their toxicity [16, 17]. In this study, we investigated the genotoxicity of TiO_2, ZnO NPs and their mixture using the *Allium cepa* assay. This study is most likely the first to investigate the interactive cytogenotoxicity and recovery effect of the mixture of TiO_2 and ZnO NPs by determining the MI and CA in *A. cepa* root cells.

Table 1 Mitotic index and frequency of chromosomal aberrations in meristematic cells of *Allium cepa* exposed to titanium dioxide and zinc oxide nanoparticles and their mixture (1:1)

Conc. (mg L^{-1})[a]	TiO_2 NPs			ZnO NPs			Mixture (1:1)			
	NDC	Mitotic index	Frequency of aberrant cell	NDC	Mitotic index	Frequency of aberrant cell	NDC	Mitotic index	Frequency of aberrant cell	Interaction factor
Genotoxicity study										
DW	220	55.0	0.14 ± 0.06	220	55.0	0.14 ± 0.06	220	55.0	0.14 ± 0.06	
5	327	81.8	0.58 ± 0.29*	51	12.8*	0.13 ± 0.10	59	14.8*	0.20 ± 0.20	− 0.37 ± 0.37
10	158	39.5	0.50 ± 0.22	69	17.3*	0.35 ± 0.44*	225	56.3	1.03 ± 0.56*	0.32 ± 0.75
20	142	35.5	0.75 ± 0.26*	85	21.3*	0.25 ± 0.26*	58	14.5*	0.55 ± 0.10*	− 0.31 ± 0.39
40	126	31.5	0.50 ± 0.37*	62	15.5*	0.38 ± 0.33*	95	23.8*	0.53 ± 0.51*	− 0.21 ± 0.71
80	107	26.8*	0.45 ± 0.34	58	14.5*	0.30 ± 0.13*	66	16.5*	0.58 ± 0.23*	− 0.03 ± 0.44
Recovery study										
DW	184	46.0	0.10 ± 0.03	184	46.0	0.10 ± 0.03	184	46.0	0.10 ± 0.03	
5	194	48.5	0.48 ± 0.35*	99	24.8*	0.25 ± 0.21*	99	24.8	0.73 ± 0.49*	0.01 ± 0.64
10	135	33.8	0.30 ± 0.18*	58	14.5*	0.18 ± 0.21*	100	25.0	0.65 ± 0.58*	0.27 ± 0.64
20	96	24.0	0.33 ± 0.28*	62	15.5*	0.35 ± 0.30*	63	15.8*	0.25 ± 0.17*	− 0.33 ± 0.45
40	126	31.5	0.43 ± 0.29*	78	19.5*	0.48 ± 0.19*	90	22.5*	0.10 ± 0.08	− 0.71 ± 0.36
80	77	19.3*	0.15 ± 0.10	144	36.0*	0.68 ± 0.45*	71	17.8*	0.60 ± 0.52*	− 0.15 ± 0.70

DW negative control (distilled water), *NDC* number of dividing cells,

[a]Total number of cells scored per concentration and in the negative control = 4000

*Significant difference from negative control ($p < 0.05$)

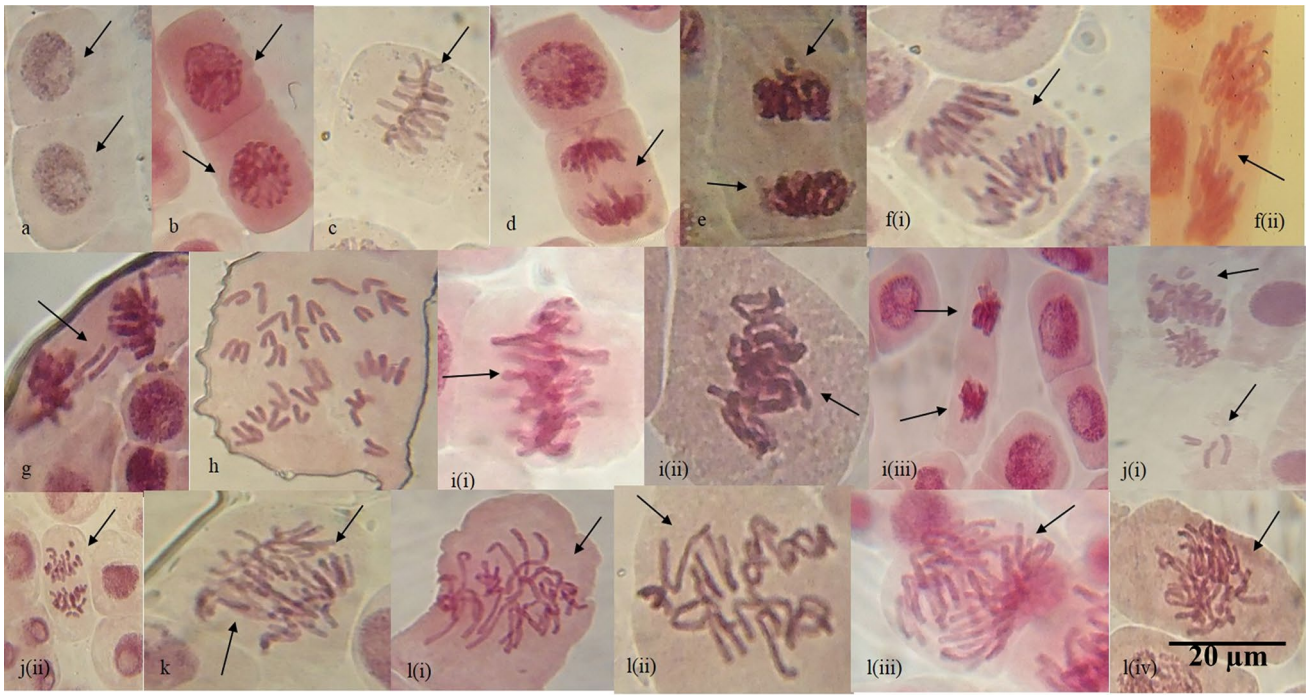

Fig. 1 Chromosome aberrations induced in *Allium cepa* root tip cells by titanium dioxide and zinc oxide nanoparticles and their mixture (1:1) **a**–**e**: normal mitotic stages in *Allium cepa* **a** interphase, **b** prophase, **c** metaphase, **d** anaphase, **e** telophase, **f**{i–ii} anaphase bridges, **g** lagging chromosomes, **h** c-mitosis, **i** sticky chromosome at {i–ii} metaphase and {iii} late anaphase-telophase, **j** {i–ii} fragmented chromosomes, **k** Anaphase with multiple bridges, **l** {i–iv} chromosomes with spindle disturbance (magnification: ×1000)

The MI is a standard parameter used for assessing cytotoxicity of environmental contaminants. Both NPs and their mixture reduced MI across the different concentrations, implying that they are mitodepressive. The mitodepressive action may be due to the suppression of DNA/protein synthesis as a result of the G1 phase blockage preventing the cells from entering mitosis [10]. Reduction in MI as observed herein strongly agrees with previous studies [36, 47, 51] where they reported significant decrease in MI in *A. cepa* root cells exposed to various concentrations of TiO_2 or ZnO NPs, but is in contrast to the report of Ghosh et al. [20], who reported that TiO_2 NPs induced a non-significant increase in the MI of *A. cepa*. This contradictory result may be due to differences in the size of the nanoparticles used for the various studies, as the smallest nanoparticles may find it easier to penetrate through the plasmodesmata of the root cells while bigger molecules may not be able to penetrate.

This study reveals the genotoxic potential of TiO_2, ZnO NPs and their mixture in plant systems. The pattern of dose response with TiO_2 showed the least chromosome damage at the highest tested dose and a decrease in CAs during the recovery period. This is in line with the report of Ghosh et al. [20] where an initial increase in DNA damaging effect followed by a decrease up to the highest treatment concentration was observed. This could be due to agglomeration of the TiO_2 nanomaterial which increases with increasing concentration. In our previous study, the dynamic light scattering showed a hydrodynamic diameter of 1492 nm for TiO_2 NPs, which was higher than the nominal size of ˂ 25 nm indicating extensive agglomeration [14]. Agglomeration of the nanoparticles might have limited the free TiO_2 NPs from interacting with the plant system at the higher concentrations [20]. Also, the reduced frequency of aberration at the highest concentration may be due to reduced MI. Since the frequency of cells have been greatly reduced, the number of cells harbouring aberration declined in similar manner too.

The recovery observed in *A. cepa* root cells exposed to TiO_2 NPs is in line with a previous study [7] where various cells have been reported to recover from nanoparticles induced genotoxicity both in vivo and in vitro. The recovery suggests that the aberrations induced by TiO_2 are transient. It is possible that the induced aberrations are not compatible to survival, the cells with the aberration could not replicate or the aberration resulted in cell death and hence the damage could not be passed to the next generation. At the same time, the aberration may be such that could be repaired, hence, the damage was not inherited by subsequent generation.

ZnO NPs induced an increase in CAs at all the tested concentrations with further increase during the recovery period. This is in line with previous reports where the genotoxicity of ZnO NPs in plant system has been documented [29, 36,

51] and their solubility compared to other metal oxide NPs [9]. More so, ZnO NPs have been reported to penetrate radically into onion roots and spoil the whole cellular metabolism and stages of cell division, affecting both the cellular and chromosomal facets [19]. The persistent presence of ZnO NPs in the cells led to increasing and continual damage, hence the genotoxic effect due to exposure to ZnO can persist for a long time in the exposed cell and are capable of being passed to the next generation. This corroborates our previous study which showed no repairs in the bone marrow cells of mice exposed to ZnO NPs [14]. Plants exposed to Zn bulk metals had also been similarly reported not to recover even after a prolonged period [12].

The CAs observed in the TiO_2/ZnO mixture was not concentration-dependent and a decrease was observed during the recovery period. This shows that the aberrations induced in the cells exposed to the mixture may also be the type(s) that could be repaired or cannot be passed to the next generation. Interaction of both NPs indicated antagonism in the induction of CAs. The possible explanation for antagonism may be due to the physicochemical properties of TiO_2 NPs and ZnO NPs, most importantly the size and agglomeration. When agglomeration occurs, it changes the physicochemical characteristics of NPs and affects their bioavailability and toxicity in the cell. Kumar and Dhawan [35] reported that agglomeration occurs when the surface charge of the NP skews towards zero, reducing the repulsion between the NPs, thereby resulting to sedimentation via gravitational force. Another factor that may explain antagonism between the two NPs is the size of the NPs. TiO_2 NPs (< 25 nm) with a larger surface area may have a higher diffusion coefficient and mobility than ZnO NPs (< 100 nm), thereby penetrating faster. This may be a possible reason for the higher frequency of CAs induced by TiO_2 NPs compared with the CAs induced by ZnO NPs.

The CAs induced by TiO_2, ZnO NPs and their mixture in this study are mostly due to spindle failure (spindle disturbance, c-mitosis and lagging chromosomes) and chromatin dysfunction (anaphase bridge and stickiness). Generally, disturbed spindles are believed to be induced by cytotoxic agents as they cause irregularity of chromosome spread at different mitotic phases [1, 43]. Most times, stickiness of chromosomes is an irreversible type of damage that leads to cell death [18]. Sticky chromosomes may also be explained through other mechanisms such as contraction and condensation of chromosomes, depolymerisation of DNA [10] and partial dissolution of nucleoproteins [28]. Chromosome and/or chromatid breakage and fusion results to anaphase bridge while the risk of aneuploidy increases through lagging chromosomes [23]. The result of this study further shows the aneugenic and clastogenic effects of TiO_2 and ZnO NPs in eukaryotic model.

A limitation in this study was that the amount of bioaccumulated TiO_2 and ZnO NPs was not quantified in the plant roots. However, several in vitro and in vivo genotoxicity reports have confirmed the internalisation of various NPs including TiO_2 and ZnO in different cells [2, 11, 21, 24, 37, 39, 52, 56]. Specifically, Kumari et al. [36] reported the internalisation of ZnO NPs while Filho et al. [16] reported that of TiO_2 NPs in *A. cepa* root cells, causing deleterious effects. This internalisation of NPs may induce oxidative stress [47] which can in turn induce genotoxicity in bio system. Thus, the observed cytogenotoxicity in this study may be due to bioaccumulation of the NPs and damages from cellular defence mechanisms in *A. cepa* root cells.

The pattern of MI and CAs induced by the mixture was different from those of each of the individual NPs. For instance, the MI at the tested concentrations for the mixture was higher than that of ZnO both in the genotoxic and recovery studies but lower than that of TiO_2. However, the CAs were higher than that observed in the corresponding concentrations of ZnO and most concentrations of TiO_2. The frequency of CAs at 10, 40 and 80 mg L in the mixture is higher than that of either of the individual NPs suggesting that exposure to the mixture of both NPs poses a greater genotoxic effect than exposure to individual NPs. This is similar to previous reports that toxicity of mixture of NPs is higher than that of the constituent NPs in plant and animal systems [32, 48].

Oxidative stress has been reported to be the one of the mechanisms of TiO_2 and ZnO NPs induced genotoxicity [20, 29]. Previous reports have shown that TiO_2 and ZnO NPs generate large amount of hydroxyl and superoxide radicals that cause oxidative stress and lipid peroxidation in animal and plant cells [20, 21]. Genotoxicity of TiO_2 and ZnO NPs have also been proposed to arise from non-direct formation of reactive species. Protonation of radical of O_2 can produce the hydroperoxyl radical (^{-}OH, H_2O_2), which can convert fatty acids to toxic lipid peroxides, destroying biological membranes [56]. Our previous study established that both NPs and their mixture were involved in ROS generation and oxidative stress in somatic tissues of mice [14]. Therefore, the ability of TiO_2, ZnO NPs and mixture to penetrate into the cell membrane and distort chromosome structure and arrangement leading to damage might be through the excess production of ROS resulting to oxidative stress and cell death. This may be one of the ways in which TiO_2 and ZnO NPs induced damage in *Allium cepa* root tips.

Conclusion

Cytogenotoxicity of the individual and interactive effects of TiO_2 and ZnO NPs on *A. cepa* genetic system was investigated. Both NPs and their mixture induced chromosome aberrations and altered the cell cycle, thereby leading to reduction in the number of dividing cells; hence were genotoxic and mitodepressive in *A. cepa* meristematic cells. ZnO induced transmissible genetic damage while TiO_2 caused transient genetic damage. The genotoxic effect by the mixture is greater than by either of the two NPs. Hence, the release of several NPs into the environment as well as the simultaneous usage of different products with different NPs present in them may be of environmental and public health effect.

Compliance with ethical standards

Conflict of interest The authors have no conflict of interest.

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