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Genotoxic effects of Bismuth (III) oxide nanoparticles by Allium and Comet assay

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HIGHLIGHTS

- Genotoxic effects of BONPs were investigated.
- Allium and Comet assays were used.
- BONPs were found genotoxic.
- BONPs also increased mitotic index.
- BONPs can cause harm to humans and the environment when not used properly.

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ABSTRACT

Genotoxic effects of Bismuth (III) oxide nanoparticles (BONPs) were investigated on the root cells of *Allium cepa* by Allium and Comet assay. *A. cepa* roots were treated with the aqueous dispersions of BONPs at five different concentrations (12.5, 25, 50, 75, and 100 ppm) for 4 h. Exposure of BONPs significantly increased mitotic index (MI) except 12.5 ppm, total chromosomal aberrations (CAs) in Allium test. While stickiness chromosome laggards, disturbed anaphase–telophase and anaphase bridges were observed in anaphase–telophase cells, pro-metaphase and c-metaphase in other cells. A significant increase in DNA damage was also observed at all concentrations of BONPs except 12.5 ppm by Comet assay. The results were also analyzed statistically by using SPSS for Windows; Duncan's multiple range test was performed. These results indicate that BONPs exhibit genotoxic activity in *A. cepa* root meristematic cells.

1. Introduction

Bismuth oxide is one of the important transition metal oxides and it has been intensively studied due to their peculiar characteristics (semiconductor band gap, high refractive index, high dielectric permittivity, high oxygen conductivity, resistivity, photoconductivity and photoluminescence, etc.). Therefore, it is used such as microelectronics (Bandoli et al., 1996), sensor technology (Hyodo et al., 2000; Khairy et al., 2010), optical coatings (Schuisky and Hårsta, 1996), transparent ceramic glass manufacturing (Pan and Ghosh, 2000), nanoenergetic gas generator (Martirosyan et al., 2009), biosensor for DNA hybridization (Taufik et al., 2011), potential immobilizing platforms for glucose oxidase (Ding et al., 2010), and polyphenol oxidase (Shan et al., 2009), fuel cells (Azad et al., 1994; Jiang et al., 2009), a additive in paints (Patil et al., 2005), an astringent in a variety of medical creams and topical ointments, and for the determination of heavy metal ions in drinking water, mineral water and urine (Pauliukaite et al., 2002). In addition this, BONPs are favorable for the biomolecules adsorption than regular sized particles because of their greater advantages and novel characteristics (much higher specific surface, greater surface free energy, and good electrochemical stability, etc.) (Ding et al., 2010; Periasamy et al., 2011).

Bismuth oxide have been found to exhibit cytotoxic activity to human dental pulp cells (Min et al., 2007) and can also induce cell death (Camilleri et al., 2004). However, white mineral trioxide aggregate or white Portland cement containing 15% Bismuth oxide were not found genotoxic and cytotoxic by comet assay and trypan blue exclusion test (Zeferino et al., 2010). Bismuth oxide did not also show genotoxic activity by comet assay in human lymphocytes (Braz et al., 2008) and in murine fibroblast cells (Ribeiro et al., 2009). Moreover, some Bismuth compounds have been found to exhibit antibacterial activity (Kotani et al., 2005), antitumor activity (Tiekink, 2002), antifungal activity (Murafuji et al., 2011) and cytotoxic activity (Friebolin et al., 2005).

The plants (*Vicia faba*, *Tradescantia paludosa*, *Pisum sativum*, *Hordeum vulgare*, *Crepis capillaris* and *A. cepa*, etc.) are most widely used in general toxicity studies. Among them, *Allium* test is one of the best-established test systems in order to determine the toxicity

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in the laboratories (Fiskesjö, 1985; Rank, 2003; Saxena et al., 2005; Konuk et al., 2007; Liman et al., 2010, 2012). Because onions are easy to store and to handle, and also macroscopic and microscopic parameters can be observed easily. Moreover this system is well correlated with the data obtained from eukaryotic and prokaryotic systems (Fiskesjö, 1988).

Comet assay (single cell gel electrophoresis, SCGE) is a technically simple, highly sensitive, fast and economic test which detects even the small changes in the single and double stranded DNA structure *in vitro* and *in vivo* conditions (Singh et al., 1988; Tice et al., 2000; Duez et al., 2003; Gichner et al., 2009). Apart from *Allium* test, root meristem cells of *A. cepa* are also used for monitoring genotoxic effect of environmental chemicals by Comet assay in different laboratories (Seth et al., 2008; Chakraborty et al., 2009; Liman et al., 2011).

The aim of this work was to assess the genotoxicity of BONPs on root cells of *A. cepa* by Allium and Comet assay.

2. Materials and methods

2.1. Materials

2.1.1. Organisms

Allium cepa ($2n = 16$) onion bulbs, 25–30 mm diameter, without any treatment, were purchased from a local supermarket.

2.1.2. Chemicals

Bismuth (III) oxide bulk (CAS No. 1304-76-3, powder), BONPs (CAS No. 1304-76-3, nanopowder, 90-210 nm particle size) basic fuchsin, methyl methanesulfonate (MMS, CAS No.67-27-3), normal melting point agarose (NMPA), low melting point agarose (LMPA), di-sodium salt of ethylene diamine tetra acetic acid (EDTA), Tris buffer, ethidium bromide (EtBr), Trizma base, Tris HCl and Triton X-100, SDS were purchased from Sigma Aldrich.

2.2. Methods

2.2.1. A. cepa anaphase–telophase test

Bismuth (III) oxide bulk and BONPs were suspended directly in distilled water and dispersed by ultrasonic vibration (130 W, 20 kHz) for 30 min to prepare five required dispersions (12.5, 25, 50, 75, and 100 ppm). All concentrations were selected arbitrarily. Prior to initiating the test, the outer scales of the bulbs and the dry bottom plate were removed without destroying the root primordia. Six healthy onion bulbs were grown in the dark at room temperature (~21 °C ± 4 °C) for 24 h. After this time, they were treated with different concentrations of BONPs for 4 h. At the end of exposure, about 0.5 cm of their tips were collected and immediately fixed in aceto:alcohol (1:3) for 24 h. The roots were transferred to 70% ethyl alcohol and stored in refrigerator. The root tips were kept in 1 M HCl for about 8 min followed by staining with Feulgen dye (20–30 min). For each test group, five slides (1 root tip/slide) were prepared by squashing root tips with 45% acetic acid. Slides were randomly coded and scored blindly. The mitotic index (MI) and the frequencies of chromosomal aberrations (CA) were carried out according to Saxena et al. (2005). For MI, the different stages of mitosis were counted in a total of 5000–6000 cells (1000 cells/slide) per concentration, and expressed as a percentage. In chromosome aberration test, 100 cells in anaphase or telophase were examined for aberrations per slide if it is possible.

2.2.2. Application of the Comet assay (single cell gel electrophoresis)

Root meristem cells of *A. cepa* were exposed to similar concentrations of BONPs as used for cytogenetic analysis. 20–30 seedlings were placed in a petri dish kept on ice and spread with 500 μL of ice-cold Tris–$MgCl_2$ buffer (0.2 M Tris, pH 7.5; 4 mM $MgCl_2$–$6H_2O$; 0.5% w/v Triton X-100). The roots were immediately chopped with a fresh razor blade and isolated root nuclei collected in the buffer. Each microscope slide was pre-coated with a layer of 1% NMPA and thoroughly dried at room temperature. Next, 100 μL of 0.8% LMPA at 37 °C was mixed with 20 μL of the nuclear suspension and dropped on top of the first layer. The slides were allowed to solidify for 5 min on an ice-cooled tray and were then immersed in ice-cold lysing solution (1 M NaCl; 30 mM NaOH, 0.5% w/v SDS, pH 12.3) for 1 h. Subsequent to lysing, the slides were placed in a horizontal gel electrophoresis chamber and the DNA was allowed to unwind for 1 h in the electrophoretic buffer, containing 30 mM NaOH and 1.5 mM EDTA at pH > 12.3 electrophoresis was then conducted for 20 min at 25 V (1 V cm^{-1}) in the chamber cooled on ice. Following electrophoresis, the slides were rinsed three times with cold distilled water for neutralization and stained with 60 μL EtBr (20 μg mL^{-1}) and covered with a cover slip (Rucińska et al., 2004). For each slide, 50 randomly chosen nuclei were analyzed using a fluorescence microscope (Olympus, Japan). Three slides were evaluated per treatment and each treatment was repeated at least twice. Each image was classified according to the intensity of the fluorescence in the comet tail and was given a value of either 0, 1, 2, 3, or 4 so that the total scores of slide could be between 0 and 200 arbitrary units (Koçyiğit et al., 2005).

2.3. Statistical analysis

The results tests were analyzed statistically by using SPSS 18.0 version for Windows software. The MI, mitotic phases, CA and comet scores expressed as percents and the levels of significance in different treatment groups were analyzed Duncan multiple range tests.

3. Results

Table 1 summarizes the effect of Bismuth oxide bulk and BONPs on MI and mitotic phase in the root meristematic cells of *A. cepa* treated for 4 h. At all concentrations used in the incubations of root increased MI compared to negative control. The highest values were obtained from 75 ppm of Bismuth oxide bulk (34.11 ± 0.48), and the lowest one 12.5 ppm of BONPs (23.5 ± 0.98). The increased of MI showed statistically significant results ($p < 0.05$) except 12.5 ppm of BONPs. MI values of Bismuth oxide bulk were found higher than BONPs except 100 ppm. The increasing MI was in a dose dependent manner ($r = 0.818$, $p < 0.01$) for BONPs. The characteristic effect caused by tested preparations of BONPs was an increase of prophase index except in 12.5 ppm and metaphase index except in 25 ppm and simultaneous decrease of anaphase and telophase index except in 12.5 ppm when compared to control. Prophase index except in 50 ppm and telophase index decreased but metaphase and anaphase index except in 50 ppm increased after application of Bismuth oxide bulk. Some of the increased and decreased phase index showed statistically significant results ($p < 0.05$).

In the *A. cepa* anaphase–telophase chromosome aberration test conducted with root meristematic cells of *A. cepa* as shown in Table 2. The most frequent abnormalities were chromosome laggards, stickiness, disturbed anaphase–telophase and anaphase bridges in anaphase–telophase cells. The effect of Bismuth (III) oxide bulk and BONPs concentration on CA was significantly different ($p < 0.05$) compared to the negative control and higher than MMS. Total aberrations for Bismuth (III) oxide bulk was found higher than BONPs in a dose dependent manner ($r = 0.774$, $p < 0.01$). In addition to these anomalies, other anomalies (prometaphase, c-metaphase and binuclear cell) were also observed.

Table 1
The effects of Bismuth oxide bulk and BONPs on mitotic index and mitotic phases in the root tips of *A. cepa* after 4 h.

Concentration	(ppm)	CCN	MI ± SD	Mitotic Phases (%) ± Standard deviation (SD)[a]			
				Prophase	Metaphase	Anaphase	Telophase
Control	-	5097	22.55 ± 0.71a	83.3 ± 1.07a	4.44 ± 1.43a	3.48 ± 1.61a	8.76 ± 2.11a
MMS	10	5202	17.03 ± 2.06b	74.2 ± 5.03b	23.43 ± 4.88b	1.01 ± 0.24b	1.34 ± 0.13b
	12.5	5137	25.47 ± 1.04c	80.23 ± 2.32ace	8.09 ± 1.32c	5.85 ± 1.36d	5.81 ± 0.75c
Bismuth oxide bulk	25	5300	30.48 ± 0.96d	81.2 ± 3.26ae	9.38 ± 1.88c	4 ± 1.2ac	5.4 ± 0.93cd
	50	5261	30.88 ± 1.17d	88.24 ± 1.52d	4.33 ± 0.99a	3.25 ± 0.32a	4.17 ± 0.33d
	75	5255	34.11 ± 0.48e	78.05 ± 0.84ce	10.38 ± 1.09c	5.18 ± 0.54cd	6.37 ± 0.96c
	100	5182	30.21 ± 1.77d	76.81 ± 2.15bc	9.18 ± 1.54c	6.08 ± 1.16d	7.93 ± 0.92a
Control	-	5097	22.55 ± 0.71a	83.3 ± 1.07a	4.44 ± 1.43a	3.48 ± 1.61ac	8.76±2.11a
MMS	10	5202	17.03 ± 2.06b	74.2 ± 5.03b	23.43 ± 4.88b	1.01 ± 0.24b	1.34 ± 0.13b
BONPs	12.5	5095	23.5 ± 0.98a	73.4 ± 2.99b	6.99 ± 2.17a	5.01 ± 1.37c	14.58 ± 1.67c
	25	5273	28.10 ± 0.62c	85.08 ± 2.83a	4.42 ± 2.14a	2.92 ± 1.31a	7.56 ± 0.98ad
	50	5136	28.44 ± 2.17cd	83.66 ± 2.89a	5.37 ± 0.6a	3.1 ± 1.61a	7.85 ± 1.39ad
	75	5330	30.39 ± 2.22de	86.63 ± 1.07a	5.49 ± 0.77a	2.57 ± 0.7ab	5.31 ± 1.37d
	100	5295	31.01 ± 1.21e	80.7 ± 1.26a	4.69 ± 0.5a	3.42 ± 1.62ac	5.17 ± 0.74d

[a] Means with the same letter do not differ statistically at the level of 0.05. CCE: Counting cell number

Table 2
Percentage of chromosome aberrations of Bismuth oxide bulk and BONPs obtained for the *Allium cepa* anaphase telophase test.

	Anaphase-Telophase Anomalies %							Other Anomalies %				
Concentration	(ppm)	CCN	DAT	CL	S	AB	TA ± SD[a]	CCN	PM	CM	BNC	TA ± SD[a]
Control	-	500	2.8	4.4	4.4	-	**11.6 ± 1.51a**	5097	0.25	0.16	-	**0.41 ± 0.12a**
MMS	10	156	1.6	21.61	0.91	-	**24.12 ± 3.7b**	5202	0.15	3.02	-	**3.17 ± 0.24b**
	12.5	500	4.4	17.8	9.6	0.6	**32.4 ± 5.63c**	5137	1	0.13	-	**1.13 ± 0.4c**
Bismuth oxide	25	500	3.4	27.6	11	0.2	**42.2 ± 3.34d**	5300	1.10	0.20	-	**1.3 ± 0.24cd**
bulk	50	500	3.4	36.69	5.11	1.04	**46.25 ± 3.13de**	5261	1.28	0.13	0.02	**1.43 ± 0.26cd**
	75	500	4	36.2	6.6	1	**47.8 ± 5.49e**	5255	1.24	0.31	0.04	**1.59 ± 0.26de**
	100	500	6.4	34.2	7	1.8	**49.4 ± 3.5e**	5182	1.54	0.33	-	**1.87 ± 0.07e**
Control	-	500	2.8	4.4	4.4	-	**11.6 ± 1.51a**	5097	0.25	0.16	-	**0.41 ± 0.12a**
MMS	10	156	1.6	21.61	0.91	-	**24.12 ± 3.7b**	5202	0.15	3.02	-	**3.17 ± 0.24b**
	12.5	500	6.4	7.6	12.8	1.4	**28.2 ± 3.11c**	5095	0.49	0.47	-	**0.96 ± 0.14c**
	25	500	9.2	11.4	16.6	1.1	**38.2 ± 2.16d**	5273	0.78	0.38	-	**1.16 ± 0.23c**
BONPs	50	500	6.4	6.79	26.54	1.21	**40.94 ± 2.39d**	5136	1.09	0.56	0.08	**1.73 ± 0.2d**
	75	500	9.4	11.67	19.39	0.6	**41 ± 4.3d**	5330	1.26	0.71	0.02	**1.96 ± 0.3de**
	100	500	10.2	15.95	14.3	0.4	**40.85 ± 1.99d**	5295	1.84	0.26	-	**2.1 ± 0.19e**

[a] Means with the same letter do not differ statistically at the level of 0.05. SD: Standard Deviation, CCN: Counting Cell Numbers, DAT: Disturbed Anaphase–Telophase, CL: Chromosome Laggards, S: Stickiness, AB: Anaphase Bridge, TA: Total Anomalies, PM: Pro-metaphase, CM: C-metaphase, and BNC: Binuclear cell.

While the lowest anomalies were observed 0.96 ± 0.14% at the 12.5 ppm, the highest one were observed 2.1 ± 0.19% at the 100 ppm for BONPs. Statistically significant ($p < 0.05$) frequencies of other anomalies were recorded for all test concentrations. The increasing total anomalies were in a dose dependent manner for Bismuth (III) oxide bulk ($r = 0.701$, $p < 0.01$) and BONPs ($r = 0.89$, $p < 0.01$).

Results obtained from Comet assay are summarized in Table 3. As it can be seen, exposure of BONPs increased the DNA damage at all concentrations in a dose dependent manner ($r = 0.936$, $p < 0.01$). Comet assay results showed that DNA damage was significantly high at all concentrations except 12.5 ppm compared to negative control. Positive control (MMS) showed highest genotoxic activity (130 ± 3.46), as compared to other treatments.

4. Discussion

Allium test was suggested to be a good indicator for assessment of nanoparticle genotoxicity, and sufficiently sensitive for application to nanostructured systems (De Lima et al., 2010; Klančnik et al., 2011; Kumari et al., 2011). The cytotoxicity levels of an agent can be determined by the increase or decrease in the MI (Fernandes et al., 2007). MIs lower than the negative control may indicate that the growth and development of exposed organisms have been affected by test compounds. On the other hand, MIs above those of the negative control are result of the induction of increased cell division, which may characterize an event detrimental to cells, leading to uncontrolled proliferation and even tumor formation (Hoshina, 2002). At all concentrations used in the incubations of root increased MI compared to negative control and the increased of MI showed statistically significant results ($p < 0.05$) except 12.5 ppm of BONPs. The increased cell proliferation activity can be the consequence of a reduction of the time necessary for DNA

Table 3
Detection of DNA damage in nuclei of A. cepa root meristems exposure to BONPs using the Comet assay.

Compounds	Concentration (ppm)	DNA Damage (Arbitrary Unit ± SD)[a]
Negative control	-	34 ± 5.29a
MMS	10	130 ± 3.46b
	12.5	42 ± 3.46a
	25	62.66 ± 5.77c
BONPs	50	70 ± 2c
	75	79.33 ± 5.03d
	100	121.6 ± 5.85e

[a] Means with the same letter do not differ statistically at the level of 0.05. SD: Standard Deviation.

repair (Evseeva et al., 2003). The increase of prophase index (except 12.5 ppm) and simultaneously decrease of both anaphase index and telophase index (except 12.5 ppm) were counted by exposing to the BONPs. This might be an indication of the blockage of chfr point (control point between prophase/metaphase). Scolnic and Halazonetis (2000) reported that chrf defines a checkpoint that delays entry into metaphase in response to mitotic stress. The effect of Bismuth (III) oxide bulk and BONPs concentration on CA was significantly different ($p < 0.05$) compared to the negative control and higher than MMS. Total aberrations for Bismuth (III) oxide bulk was found higher than BONPs. Among above aberrations, disturbed anaphase–telophase (especially 100 ppm) and chromosome laggards could occur by the effect of BONPs and Bismuth (III) oxide bulk on microtubule formations (Amer and Ali, 1986; Kumari et al., 2009). Such spindle malfunctioning may arise due to inhibition of tubulin polymerization (Kuriyama and Sakai, 1974). The occurrence of chromosome laggards at anaphase was due to the failure of the chromosomes or acentric chromosome fragments to move to either of the pole. Stickiness (especially at 50 and 75 ppm) indicates highly irreversible type toxic effect of BONPs, and its occurrences during the study could be because of sub-chromatid linkage between chromosomes (Mc-Gill et al., 1974; Chauhan et al., 1986; Ajay and Sarbhoy, 1988; Kovalchuk et al., 1998). Anaphase bridges could happen during the translocation of the unequal chromatid exchange or due to dicentric chromosome presence or due to the breakage and fusion of chromosomes and chromatids. This bridges cause structural chromosome mutations (El-Ghamery et al., 2000; Luo et al., 2004). C-metaphase, a possibly reversible effect, might be occurred due to disturbed microtubules by Bismuth (III) oxide and could be induced aneuploidy (Fiskesjö, 1988; Shahin and El-Amoodi, 1991; Odeigah et al., 1997). Some of the nanoparticles were also found genotoxic in Allium test, i.e. silver nanoparticles (Kumari et al., 2009), nano-TiO_2 (Ghosh et al., 2010), zinc oxide nanoparticles (Kumari et al., 2011), multi-walled carbon nanotubes (Ghosh et al., 2011) and in different chromosomal aberration tests, i.e. ferrofluid (Racuciu and Creanga, 2007), acridines (Di Giorgio et al., 2008), aluminum oxide nanomaterials (Balasubramanyam et al., 2009a), CeO_2 (Lopez-Moreno et al., 2010), silver nanoparticles (Wise et al., 2010; Patlolla et al., 2012), silica nanoparticles (Park et al., 2011; Lankoff et al., 2012).

BONPs induced a dose dependent increase in extent of DNA damage with significant ($p < 0.05$) damage observed at concentrations above 12.5 ppm. Positive results were also obtained from comet assays with other nanoparticles (Martino-Roth et al., 2003; Kisin et al., 2007; Balasubramanyam et al., 2009b; Gopalan et al., 2009; Grigg et al., 2009; Sharma et al., 2009; Migliore et al., 2010; Hackenberg et al., 2010, 2011; Asare et al., 2012). The DNA damaging activity of BONPs could be attributed to a property of nanomaterials to form agglomerates by virtue of which, with increase in treatment concentration the nanoparticles had a tendency to precipitate (Ghosh et al., 2010) or could be associated with the generation of free radicals. Free radicals can be released after nanoparticles exposure because they have a large surface area to mass ratio, and are highly reactive when they are exposed to aqueous media or cellular components or nanoparticles may adsorb free radicals onto their surface in addition to releasing free radicals (Flower et al., 2012). Free radicals may induce: the cross linking between DNA and proteins, the damage between deoxyribose and phosphate skeleton, and specific modifications between purine and primidine bases, single or double strand breaks (Dizdaroğlu, 1991; Shigenaga and Ames, 1991). These oxidation products may cause the mutations via to the release of base and induces fracture of DNA chain (Kuchino et al., 1987; Cheng et al., 1992).

As a result, BONPs were a genotoxic effect by increasing CAs and DNA damage and it also induced cell division by increasing the MI in *A. cepa* root meristematic cells. In our knowledge, this is the first genotoxicity study of BONPs. Therefore further studies should be conducted to better understand the molecular mechanisms involved in the genotoxicity of BONPs for its safer and proper utilization.

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