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Genotoxic evaluation of different sizes of iron oxide nanoparticles and ionic form by SMART, Allium and comet assay

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Abstract

In this study, the genotoxic potential of <50 nm, <100 nm iron oxide (Fe_2O_3) nanoparticles (IONPs) and ionic form were investigated using the wing somatic mutation and recombination test (SMART) and Allium and comet assays. In the SMART assay, different concentrations (1, 2, 5 and 10 mM) of NPs and ionic forms were fed to transheterozygous larvae of *Drosophila melanogaster*. No significant genotoxic effect was observed in <100 nm NPs and ionic form, while <50 nm IONPs showed genotoxicity at 1 and 10 mM concentrations. *Allium cepa* root meristems were exposed to five concentrations (0.001, 0.01, 0.1, 1 and 10 mM) of <50 nm and ionic forms for 4 h and three concentrations (2.5, 5 and 10 mM) for <100 nm of IONPs for 24 and 96 h. There was a statistically significant effect at 96 h at all concentrations of <100 nm IONPs. Similarly, <50 nm of IONPs and ionic forms also showed a statistically significant effect on mitotic index frequencies for all concentrations at 4 h. There was a dose-dependent increase in chromosomal abnormalities for IONPs and ionic form. Comet assay results showed time- and concentration-dependent increases in <100 nm NPs. There was a concentration-dependent increase in <50 nm NPs and ionic form ($p < 0.05$). Consequently, the <50 nm of Fe_2O_3 was found toxic compared to 100 nm Fe_2O_3 and ionic form.

Keywords

Fe_2O_3 nanoparticles, SMART, Allium test, comet test, genotoxicity

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Introduction

Nanotechnology has revolutionized human society, particularly the medicine and cosmetics industry. This development is related to the many unique and valuable characteristics of the nanomaterials. However, these properties could be an issue of concern because of uncontrolled use and release into the environment. This rapid development of new nanomaterials raises questions about their impact on the environment and human health (Golbamaki et al., 2015).

Nowadays, iron oxide (Fe_2O_3) nanoparticles (IONPs) are extensively being used for several purposes, like radiological diagnostic procedures, detoxification of biologic fluids, immunoassay, and drug delivery, for example (Bellin et al., 2000; Marchal et al., 1989; Nanz et al., 2000; Saini et al., 2000). But, its toxicity is still a concern as different sizes of NPs behave differently.

Therefore, there are efforts to work further in this area to make non-biodegradable NPs to more biocompatible. Different test systems are used to evaluate the genotoxicity of different sizes of IONPs. Demir et al. (2011) first suggested the wing somatic mutation and recombination test (SMART) in *Drosophila melanogaster* as a useful model for detecting the

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genotoxic potential of NPs. The SMART has provided a cheap, rapid and sensitive assay, which is able to detect variations of genetic mutations and somatic recombination. The alkaline comet assay is considered a very promising tool to detect genotoxicity of any toxic compound. Studies by, Fiskesjö (1985) and Chauhan and Gupta (2005) demonstrated high efficiency of the *Allium cepa* test to indicate chromosomal damage and disorders of the mitotic cycle, due to its sensitivity and good correlation with mammalian tests. Fiskesjö (1985) and Carita and Marin-Morales (2008) concluded that the test can be used in genotoxicity and cytotoxicity studies and with a wide variety of environmental pollutants such as heavy metals, pesticides, aromatics and complex mixtures of pollutants. Besides environmental monitoring testing, genotoxicity with *A. cepa* is being applied in the analysis of NPs, mainly to check their safety and genotoxicity (Demir et al., 2014). Therefore, the current study combined these tests to investigate the genotoxic potential of various sizes of IONPs and ionic form.

Materials and methods

Size determination of IONPs was determined by zeta potential and transmission electron microscopy. These studies were conducted at the central laboratory of Middle East Technical University.

SMART

SMART was carried out as described by Demir et al. (2014). Five groups were made. The first three groups contained transheterozygous larvae with four different concentrations (1, 2, 5 and 10 mM) of <50 nm, <100 IONPs and ionic form, respectively. Distilled water and 1-mM ethyl methanesulfonate (EMS) were used as normal control and positive control for NPs, respectively, while ethyl alcohol (10%) was fed as a normal control for the ionic form group. The percentage of mutation inhibition was calculated by Abraham (1994), and wings were scored by the method of Graf et al. (1984).

Allium test

Healthy onion bulbs were grown inside test tubes in distilled water. *A. cepa* onion bulbs were kept in a dark room for 24 h. Different concentrations (10, 8, 6, 4, 2, 1, 0.1, 0.01 and 0.001 mM) of <100 nm IONPs were selected to establish EC_{50} arbitrarily and kept for 96 h. Distilled water was used for the negative control group. After finding the EC_{50}, five onion bulbs were taken and $2 \times EC_{50}$, EC_{50} and $½ \times EC_{50}$ concentrations were used for the Allium test for 24 and 96 h, whereas, for <50 nm IONPs and ionic form, 0.001, 0.01, 0.1, 1 and 10 mM concentrations were used and exposed for 4 h to *A. cepa* roots. Methyl methanesulfonate (MMS) and distilled water were used as positive and negative control, respectively. The mitotic index (MI) and chromosomal aberrations (CAs) were calculated as described by Cigerci et al. (2015, 2016).

Comet assay

Root meristem cells of *A. cepa* were exposed to different concentrations (0.001, 0.01, 0.1, 1 and 10 mM) of <50 nm IONPs and Fe_2O_3 ionic form for 4 h, while $2 \times EC_{50}$, EC_{50} and $½ \times EC_{50}$ concentrations of <100 nm IONPs were exposed for 24 and 96 h to perform the comet assay. After the exposure time, root tips were cut, crushed and kept in ice-cold tris-magnesium chloride buffer. Then, 100-µl isolated root nuclei mixed with 20-µl long melting point agar (LMPA) and spread on already 1% normal melting point agar (NMPA) pre-coated slide. The slides were kept on cold slabs for 3–5 min and then put in lysing solution at 4°C for 1 h (Cigerci et al., 2015, 2016). After lysing, slides were kept in an electrophoresis solution for 20 min and then the electrophoresis chamber was run for at 25 V (1 V cm^{-1}) for 20 min at 4°C. Subsequent to electrophoresis, slides were neutralized with chilled distilled water, stained with ethidium bromide (60 µl) (Rucinska et al., 2004) and observed under a fluorescence microscope (Collins, 2004). Intensity of DNA damage was calculated by the comet length as described by the Cigerci et al. (2015, 2016).

Statistical analysis

The results obtained from SMART were evaluated by the MICROSTA program. Statistical analysis of all other results was done by the analysis of variance of the data using SPSS version 15.0 for Windows software. Duncan's multiple range test was used for comparing different concentrations groups with the controls.

Results

Results of transmission electron microscopy for <50 and <100 nm IONPs are shown in Figures 1 and 2. Both figures revealed the existence of different shapes of particles: oval shaped and worm shaped of <50 nm

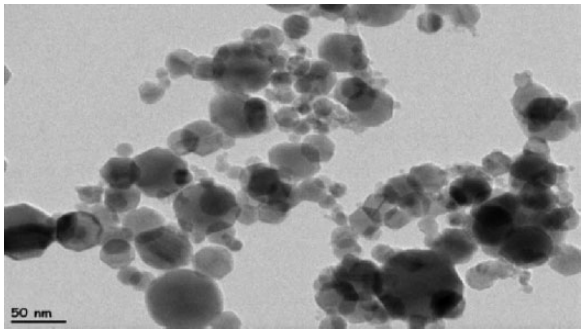

Figure 1. <50 nm IONPs analyzed by the transmission electron microscope. IONP: iron oxide nanoparticle.

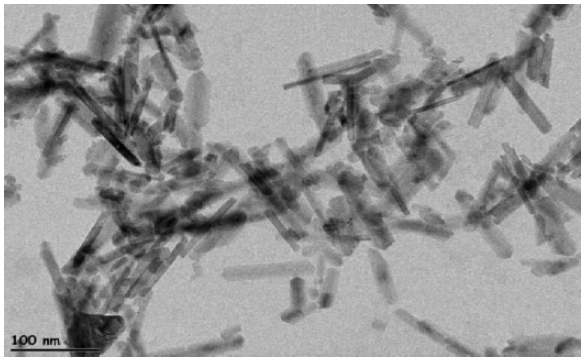

Figure 2. <100 nm IONPs analyzed by the transmission electron microscope. IONP: iron oxide nanoparticle.

IONPs, whereas roughly elongated and rod-shaped (smooth longer gathering of magnetic cores) of <100 nm IONPs and magnetic bead (arbitrary distribution of IONPs in polymeric beads) were observed.

Results obtained from SMART for the NPs and ionic form are summarized in Tables 1 and 2. Different concentrations (1, 2, 5 and 10 mM) of NPs and ionic form were fed to transheterozygous larvae. No significant genotoxic effect was observed in <100 nm NPs and ionic form, while <50 nm IONPs showed genotoxicity at 1 and 10 mM concentrations.

Increase in root length was observed at 1 mM, then it started to decrease. Growth inhibition was found at 10 mM. MI and mitotic phase frequencies were calculated separately for each concentration and duration (Tables are provided as Online Supplementary material). Effect on the MI for <100 nm IONPs for 24 and 96 h at 5 mM concentration was observed, while there was a statistically significant effect for 96 h at all concentrations of <100 nm of IONPs. Similarly, <50 nm of IONPs and ionic form also showed a statistically significant effect on MI frequencies for all concentrations at 4 h. A statistical significant difference ($p < 0.05$) for MI was found for the <100 nm IONPs at 5 mM concentration compared to control and MMS groups. There was a concentration-dependent increase in MI for <50 nm

Table 1. Results of SMART, evaluating the genotoxic potential of IONPs.

Experimental groups	Number of wings (*N*)	Small single spots (1–2 cells; $m = 2$)			Large single spots (>2 cells; $m = 5$)			Twin spots ($m = 5$)			Total mwh spots ($m = 2$)			Total spots ($m = 2$)			Frequency of clone formation (10^5 cells)
		No.	Fr.	D.	No.	Fr.	D.	No.	Fr.	D.	No.	Fr.	D.	No.	Fr.	D.	
Normal wing																	
Distilled water (72 h)	80	13	0.16		2	0.02		0	0.00		15	0.19		15	0.19		0.76
1 mM EMS (72 h)	31	71	2.29	+	27	0.87	+	9	0.29	+	105	3.39	+	108	3.48	+	5.38
72 ± 4 h < 50 nm Fe_2O_3 (mM)																	
1	80	22	0.27	i	5	0.06	i	1	0.01	i	27	0.33	+	28	0.35	+	1.38
2	80	20	0.25	i	5	0.06	i	0	0.00	i	25	0.31	i	25	0.31	i	1.28
5	80	14	0.17	i	6	0.07	i	1	0.01	i	20	0.25	i	21	0.26	i	1.02
10	80	31	0.38	+	3	0.03	i	2	0.02	i	31	0.38	+	36	0.45	+	1.59
72 ± 4 h < 100 nm Fe_2O_3 (mM)																	
1	80	15	0.18	i	5	0.06	i	0	0.00	i	20	0.25	i	20	0.25	i	1.02
2	80	6	0.07	−	2	0.02	i	0	0.00	i	8	0.10	−	8	0.10	−	0.41
5	80	1	0.01	−	2	0.02	i	0	0.00	i	3	0.03	−	3	0.03	−	0.15
10	80	6	0.07	−	2	0.02	i	2	0.02	i	10	0.12	−	10	0.12	−	0.51

Fr.: frequency; D.: statistical results; +: positive; −: negative; i: non-significant results; m: multiplying factors; confidence level: 0.05; SMART: somatic mutation and recombination test; IONP: iron oxide nanoparticle; Fe_2O_3: iron oxide.

Table 2. Results of SMART, evaluating the genotoxic potential of Fe_2O_3 ionic form.

Experimental groups	Number of wings (*N*)	Small single spots (1–2 cells; $m = 2$)			Large single spots (>2 cells; $m = 5$)			Twin spots ($m = 5$)			Total mwh spots ($m = 2$)			Total spots ($m = 2$)			Frequency of clone formation (10^5 cells)
		No.	Fr.	D.	No.	Fr.	D.	No.	Fr.	D.	No.	Fr.	D.	No.	Fr.	D.	
Normal wing																	
%10 Ethyl alcohol (72 h)	80	22	0.28		3	0.04		1	0.01		26	0.32		26	0.32		1.33
1 mM EMS (72 h)	80	67	0.84	+	24	0.30	+	9	0.11	+	86	1.08	+	100	1.25	+	4.40
Fe_2O_3 ionic form (mM)																	
1	80	33	0.41	i	2	0.02	–	1	0.01	i	36	0.45	I	36	0.45	i	1.84
2	80	18	0.22	–	5	0.06	i	0	0.00	i	23	0.29	–	23	0.29	–	1.18
5	80	25	0.31	–	0	0.00	–	0	0.00	i	25	0.31	–	25	0.31	–	1.28
10	80	17	0.21	–	2	0.02	–	0	0.00	i	19	0.24	–	19	0.24	–	0.97

Fr.: frequency; D.: statistical results; +: positive; –: negative; i: non-significant results; m: multiplying factors; confidence level: 0.05; SMART: somatic mutation and recombination test; Fe_2O_3: iron oxide.

Table 3. Different anaphase–telophase chromosomal abnormalities in *Allium cepa* root cells due to <100 nm IONPs.

Experimental groups	Total number of cells counted	Disturbed anaphase–telophase	Anaphase–telophase abnormalities (%)[a]			
			Laggards chromosomes	Stickness	Anaphase bridge	Total abnormalities (% ± SD)
Control-24 saat	500	0.6	12.4	1.6	5	19.6 ± 0.89[b]
MMS-10 mg/L	500	10.2	8	14.4	4	36.6 ± 0.81[c]
2.5 mM	500	0.4	11.4	1.4	5	18.2 ± 1.92[b]
5 mM	500	0.2	14.2	1.4	3	18.8 ± 1.78[b]
10 mM	500	0.6	13.6	2.6	2.2	19 ± 3.31[b]
Control-96 saat	500	–	15.2	1.4	3.6	20.2 ± 2.38[b]
MMS-10 mg/L	500	17.6	6.6	19.2	12	44.4 ± 1.74[c]
2.5 mM	500	0.4	19.8	1.6	2.8	24.6 ± 2.19[d]
5 mM	500	0.6	18	2.6	4.6	25.8 ± 2.28[d,e]
10 mM	500	0.6	19.2	2.2	6.6	28.6 ± 2.5[e]

IONP: iron oxide nanoparticle.
[a]Different small letters in columns showing significant level ($P < 0.05$).

NPs and ionic form after 4 h and it was found statistically significant ($p < 0.05$).

The disturbed anaphase–telophase, chromosome laggards, stickiness and anaphase bridge were observed for different sizes of IONPs and ionic form (Tables 3 and 4). The total chromosome anomalies increased with an increase in the exposure time and the concentrations of different sizes of IONPs. The total chromosome aberrations (%) were significantly greater ($p < 0.05$) at 96 h for <100 nm NPs, while statistical significance was found only for 0.1–10 mM concentrations for <50 nm NPs.

Comet assay demonstrated the extent of DNA damage caused by the different sizes of NPs (Figure 3). Comet assay results showed time- and concentration-dependent increases in DNA damage for <100 nm NPs at 24 and 96 h (Table 5). The most DNA damage was observed at 10 mM and the least was at 2.5 mM for 24 and 96 hand these were statistically different ($P < 0.05$). Similar results were observed for the <50 nm NPs and ionic form at 4 h. There was a concentration-dependent increase in DNA damage. Similarly, application of <50 nm NPs and ionic form for 4 h also showed DNA damage as the concentration increased ($p < 0.05$; Table 6).

Table 4. Different anaphase–telophase chromosomal abnormalities in *Allium cepa* root cells due to <50 nm NPs and ionic form of Fe_2O_3.

		Anaphase–telophase abnormalities (%)[a]				
Experimental groups	Total number of cells counted	Disturbed anaphase–telophase	Laggards chromosomes	stickness	Anaphase bridge	Total abnormalities (% ± SD)
Control-4 h	500	–	12	1.4	2	15.4 ± 2.07[b]
MMS-10 mg/L	142	4.2	40.22	0.87	–	45.28 ± 3.83[c]
0.001 mM	500	0.2	10.4	3.2	2.2	16 ± 2.12[b]
0.01 mM	500	0.2	11.8	3.2	2.6	17.8 ± 1.78[b]
0.1 mM	500	0.6	18.6	5	2.8	27 ± 3.74[d]
1 mM	500	0.2	18.6	7	4.8	30.6 ± 2.3[e]
10 mM	500	–	27.4	9.4	3	39.8 ± 1.92[f]
Ionic form of Fe_2O_3						
Control 4 h	500	–	12	1.4	2	15.4 ± 2.07[b]
MMS-10mg/L	142	4.2	40.22	0.87	–	45.28 ± 3.83[c]
0.001 mM	500	–	1.8	2.8	–	4.6 ± 1.51[d]
0.01 mM	500	0.2	3.4	1.4	0.2	5.2 ± 1.3[d]
0.1 mM	500	–	3.2	0.2	2	5.4 ± 1.51[d]
1 mM	500	0.4	6.6	0.6	1.2	8.8 ± 1.3[e]
10 mM	500	–	2.2	2.2	5	9.4 ± 1.67[e]

NP: nanoparticle; SD: standard deviation; Fe_2O_3: iron oxide.
[a]Different small letters in columns showing significant level ($p < 0.05$).

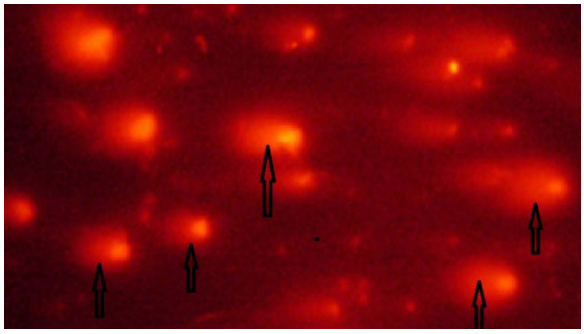

Figure 3. DNA damage caused by the exposure of NPs. Arrows are indicating the tail. The tail length shows the degree of DNA damage. NP: nanoparticle.

Table 5. DNA damage effects on the *Allium cepa* root meristems by the <100 nm Fe_2O_3.[a]

		DNA damage (arbitrary unit)	
		Average ± standard deviation	
Groups	Concentrations	24 h	96 h
Negative control	–	11 ± 1.73[b]	14 ± 1.73[b]
MMS	10 ppm	23.33 ± 1.52[c]	30 ± 2[c]
	2.5 mM	16.33 ± 2.08[d]	20.66 ± 1.52[d]
	5 mM	19.33 ± 1.15[d]	28 ± 2.64[c]
	10 mM	23.33 ± 2.3[c]	32 ± 3.46[c]

Fe_2O_3: iron oxide.
[a]Different small letters in columns showing significant level ($p < 0.05$).

Discussion

Nowadays, extensive studies are being conducted to evaluate the potential genotoxic risks due to exposure to emerging nanomaterials. This study is unique in the sense that different test systems were employed to compare the genotoxicity of different sizes of IONPs.

The SMART test indicated no significant genotoxic effect for <100 nm NPs and ionic form, while <50 nm IONPs showed genotoxicity at lower and higher concentrations. SMART is an *in vivo* sensitive assay, which is based on the loss of heterozygosity of two genetic markers of mwh and flare3 (flr3). These markers are mainly involved in the metabolic pathways of these two strains. Clone(s) of mwh and/or flr3 cells become visible on the wings of adult fly, which is caused by the genetic changes in the mitotic cells of a developing wing disc (Graf et al., 1984). In the current study, no obvious genetic mutation was observed by the different sizes of IONPs except <50 nm. It could be due to the minor DNA damage and may be repaired and thus cannot be detected by SMART.

Table 6. DNA damage Effects on the *Allium cepa* root meristems by the <50 nm NPs and ionic form of Fe_2O_3.[a]

Experimental groups	Concentrations	DNA damage (arbitrary unit) average ± standard deviation
Negative control	–	13.33 ± 1.15[b]
MMS	10 ppm	24.66 ± 1.52[c]
	0.001 mM	15.33 ± 1.15[b,d]
	0.01 mM	18.66 ± 2.3[d]
	0.1 mM	21 ± 3.6[c]
	1 mM	22.33 ± 2.08[c]
	10 mM	32.66 ± 4.16[e]
Negative control for ionic form	–	13.33 ± 1.15[b]
MMS	10 ppm	24.66 ± 1.52[c]
Ionic form of Fe_2O_3	0.001 mM	12.4 ± 2.6[b]
	0.01 mM	14.33 ± 1.51[b]
	0.1 mM	16.66 ± 3.02[d]
	1 mM	18.33 ± 3.13[d]
	10 mM	24.06 ± 2.68[c]

MMS: methyl methanesulfonate; NP: nanoparticle; Fe_2O_3: iron oxide.
[a]Different small letters in columns showing significant level ($p < 0.05$).

A concentration-dependent increase in the inhibition of root growth and the appearance of stunted roots indicated cytotoxicity of IONPs and ionic form. It demonstrated that IONPs and its ionic form have an effect on root elongation, cell morphology and the adsorption potential in a hydroculture of *A. cepa*. Moreover, the phytotoxic nature of these NPs could be due to the heavy accumulation in the cellular and the chromosomal components (Demir et al., 2014). Increase in MI, mitotic phase frequencies and chromosome aberrations was also observed in a dose-dependent manner for IONPs except at 5 mM concentration of <100 nm IONPs for 24 h. *A. cepa* and anaphase–telophase chromosome aberration assay are standardized and highly reliable tests to study cytotoxicity and genotoxicity (Fiskesjö, 1985; Konuk et al., 2007; Yıldız and Arıkan, 2008). Different chromosome anomalies were observed by the IONPs which generally show the toxic effects of these NPs.

The impaired anaphase–telophase and total CA may be due to the effects of IONPs on microtubule formation (Amer and Ali, 1983). Stickiness could be caused by the sub-chromatid linkage between chromosomes (Mc-Gill et al., 1974) or chromosomes unable to move due to presence of IONPs and get stuck somewhere (Ajay and Sarbhoy, 1988). This is also explained as chromosomal proteins got physical adhesions and chromosomes could not move to the final destination (Patil and Bhat, 1992). Anaphase bridges were also observed in the current study, which could be due to the dicentric chromosome presence. These bridges are the source of structural chromosomal mutations (El-Ghamery et al., 2000). Likewise, *A. cepa* assay has also proved an efficient test to demonstrate toxicity for the other different NPs (Liman, 2013). Recent studies showed that IONPs can be cytotoxic and clastogenic/genotoxic in nature. Moreover, it could also be proposed that IONPs penetrated into the plant system and damaged the stages of cell division, thus causing the disturbed metaphase, chromatin bridge, stickiness, multiple chromosomal break and so on.

Comet assay showed the time- and dose-dependent increase in DNA damage for <100 nm nanoparticles, and there was a concentration-dependent increase observed in <50 nm nanoparticles and ionic form. Genotoxic and cytotoxic effects of IONPs had been found in various cell lines (Ahamed et al., 2013; Magdolenova et al., 2015; Valdiglesias et al., 2015). It was also evident by previous studies that IONPs induce oxidative stress in a dose-dependent manner, by the depletion of glutathione, lipid peroxidation and production of reactive oxygen species. Comet assay demonstrated the higher level of DNA damage with higher concentrations of IONPs in skin and lung epithelial cell lines (Ahamed et al., 2013). Regardless of the positive correlation between IONPs exposure and genotoxicity as described above, negative association for IONPs genotoxicity has also been found in many other studies (Karlsson et al., 2008; Singh et al., 2013). It is obvious from these studies that the genotoxic potential of IONPs is mainly due to their ability to induce DNA breaks and oxidative DNA damage. It seems that this ability may be greatly influenced by IONPs characteristics such as size or surface coating nature. These controversial results of IONPs due to particle size and surface coating are indicative of cellular responses, intensity of effects and potential mechanisms of toxicity by these NPs (Valdiglesias et al., 2015).

This demonstrates that particle size seems to be crucial for iron oxide–induced genotoxicity, as different sizes of NPs may behave differently and have different genotoxic potential. The genotoxic potential of NPs could be different, which is mainly related to their sizes, surface topology, physical properties and

different concentrations (Balasubramanyam et al., 2009). In the current study, the results were conflicting with respect to different doses and among different assay systems but overall, <50 nm Fe_2O_3 showed more genotoxic potential compared to other sizes.

To the best of our knowledge, this study is the first to assess the different sizes of genotoxic potential of IONPs by SMART and Allium test.

Conclusion

It is concluded that comparatively, <50 nm Fe_2O_3 showed genotoxic potential as compared to <100 nm and ionic form of Fe_2O_3, and particle size seems to be crucial for the observed iron oxide–induced genotoxicity. It could be deduced that NPs especially <50 nm could be genotoxic because of its smaller size and large surface area.

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Supplemental material

Supplementary material for this article is available online.

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