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CYTOTOXICITY AND GENOTOXICITY OF IRON OXIDE NANOPARTICLES: AN *IN VITRO* BIOSAFETY STUDY

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Abstract: With the development of nanotechnology and the wide use of iron oxide nanoparticles, it has become necessary to assess the potential adverse biological effects of magnetite. This study investigated the cytotoxicity, genotoxicity and oxidative damage of different concentrations of magnetite (0 to 1000 mg/L) in human whole blood cultures. After supplementation of magnetite, the blood samples were incubated for 72 h. Cell viability was assessed by the 3-(4,5-dimethylthiazol-2-yl) 2,5-diphenyltetrazolium bromide (MTT) and lactate dehydrogenase (LDH) release assays. The total antioxidant capacity (TAC) and total oxidant status (TOS) were determined to evaluate the dose-dependent effects of magnetite on the oxidant/antioxidant balance and to evaluate the potential oxidative injury due to increased oxidative stress. Genotoxicity was estimated by by the sister chromatid exchange (SCE), micronuclei (MN) and chromosome aberration (CA) assays and determination of 8-oxo-2-deoxyguanosine (8-OH-dG) levels. The results of MTT and LDH assays showed that the higher concentrations of magnetite (100, 150, 300, 500 and 1000 mg/L) decreased cell viability. Concentrations of magnetite higher than 10 mg/L increased TOS levels and decreased TAC levels in human blood cells. Increasing concentrations of magnetite caused significant increases in MN, SCE and CA rates and 8-OH-dG levels. The obtained results showed that magnetite exerted dose-dependent effects on oxidative damage, genotoxicity and cytotoxicity in human blood cells.

Key words: Cytotoxicity; genotoxicity; iron oxide; lymphocyte; oxidative stress

INTRODUCTION

Nanoparticles (NPs) are identified as particles with diameters under 100 nm. The rapidly developing area of nanotechnology has brought about a veritable plethora of novel NPs for diverse applications, ranging from solar energy capture to cosmetics and drug delivery (Riehemann et al., 2009). In recent years, with the development of nanotechnology, more and more nanocomposites have been designed and manipulated to make specific functions, such as adhesives, drug delivery and artificial organs and tissues (Dvir et al., 2011). With increasing commercialization and use of nanomaterials, there is an increased exposure to NPs through inhalation, ingestion, skin uptake and injection of engineered nanomaterials (Sundaram et al., 2006). Therefore, researching the toxicity of NPs

is of importance to provide guidance to occupational health and safety (Li et al., 2008; Lanone et al., 2009).

To date, a range of efforts have been made to discover new therapeutic substances, such as vitamins, fatty acids, lichen metabolites, medicinal plant extracts, polysaccharides, various antioxidants, mushroom extracts, amino acid derivatives, antibodies, microbial biofilms. Recently, several nanoparticles have been used as effective carriers of such therapeutic substances (Cingolani et al., 2000; Cacciatore et al., 2003; Rispoli et al., 2004; Cacciatore et al., 2005; Di Stefano et al., 2009; Heuking et al., 2009; Turkez et al., 2005; Turkez et al., 2010; Sozio et al., 2010; Turkez et al., 2012; Turkez and Aydin, 2013; Alimpic et al., 2014; Grujic et al., 2014; Milovanovic et al., 2014). At present, biosafety evaluations of these newly explored or engineered materials are considered necessary for their safe usage, and investigating the toxicity of NPs is of importance to provide guidance to occupational health and safety (Li et al., 2008; Lanone et al., 2009).

Magnetite NPs are one of the most important members of this class of materials, which have found extensive application in biomedical and biotechnological fields. Magnetic NPs like magnetite, due to their unique magnetic and electronic properties, are used in various applications such as biomedical drug delivery, gene therapy, bioprocessing, cell and tissue engineering, specific site targeting, magnetic data storage, information storage, sensors, catalysis, ferro fluids and energy storage (Lewin et al., 2000; Xiang et al., 2003; Jain et al., 2005; Haubner et al., 2010; Xie et al., 2012; Metin et al., 2012). Thus, the possible health impact of magnetite NPs upon introduction into the body is of great interest. With the increased application of magnetite NPs, concerns about their potential human toxicity and their environmental impact have also increased. However, toxicity data for magnetite NPs in relation to human health are limited and only a few results from basic studies have been published. Some studies provided ample evidence that the cytotoxicity of magnetite NPs may be partially due to their induction of cellular oxidative stress through the production of reactive oxygen species (ROS) (Apopa et al., 2009). Furthermore, it has been reported that different physical and chemical properties of particles, including magnetite, have the potential to influence the interaction with biological systems (Könczöl et al., 2011; Kawanishi et al., 2013).

Since increased ROS formation and oxidative stress are important factors in the development of cytotoxicity, in the present study specific measurements of these parameters were made in human lymphocytes. Different aqueous magnetite concentrations (5, 10, 20, 50, 75, 100, 150, 300, 500 and 1000 ppm) were investigated. All samples were evaluated for their genotoxic, cytotoxic effects and capability for ROS generation in cellular systems.

MATERIALS AND METHODS

Synthesis of magnetite (<20 nm)

Fe_3O_4 magnetic NPs were prepared by the thermal decomposition of iron acetylacetonate ($Fe(acac)_3$) in the presence of oleylamine and benzyl ether (Caldıran et al., 2013). Oleylamine (>70%), benzyl ether, $Fe(acac)_3$ (99%) and hexane (99%) were purchased from Sigma-Aldrich and used without further purification. Phase and textural analysis was undertaken using X-ray diffraction (XRD) and X-ray rucking curves with CuK_α (Bruker AXS D8-Advanced) (λ=1.5418 Å), at 30 kV, 10 mA. Fig. 1 shows the XRD pattern of as-prepared Fe_3O_4 NPs. The position and relative intensity of all diffraction peaks are consistent with Fe_3O_4 NPs in the literature, which confirms the magnetite structure (Xu et al., 2009). In addition, Fig. 2 shows the transmission electron microscope (TEM) image of monodisperse Fe_3O_4 NPs that were examined by TEM analysis (JEOL 2100 TEM (200 kV)).

Experimental design

The studies were approved by the appropriate local committees, and were performed according to the Declaration of Helsinki. Blood samples were obtained from six healthy non-smoking donors. Questionnaires were completed by each blood donor to evaluate exposure history, and each donor signed in-

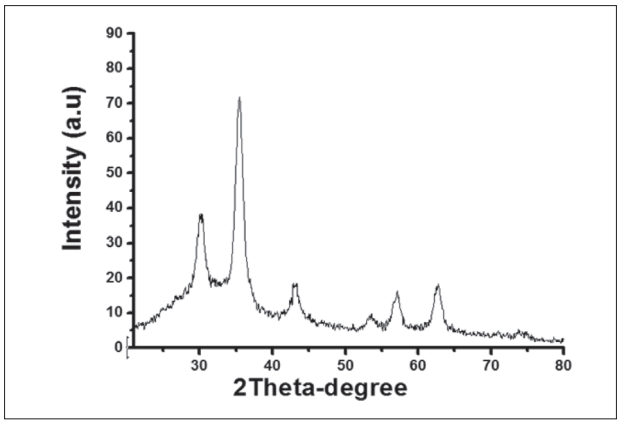

Fig. 1. The XRD pattern of as-prepared Fe_3O_4 NPs.

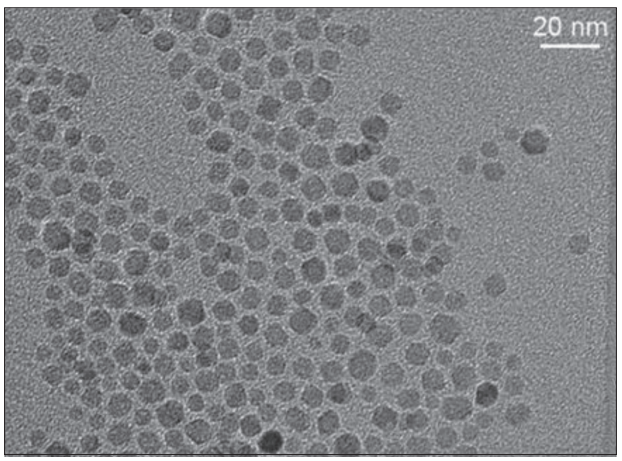

Fig. 2. Representative TEM images of as-prepared Fe_3O_4 NPs.

formed consent forms. In all the volunteers involved in this study, hematological and biochemical parameters were analyzed and no pathology was detected. Human peripheral blood lymphocyte cultures were set up according to the protocol described by Evans and O'Riordan (1975). The heparinized blood (0.5 ml) was cultured in 6 ml of culture medium (Chromosome Medium B, Biochrom®, Leonorenstr. 2-6, D-12247, Berlin) with 0.005 mg/L of phytohemagglutinin (Biochrom®). Different aqueous magnetite concentrations (5, 10, 20, 50, 75, 100, 150, 300, 500 and 1000 mg/L) were added into culture tubes. After supplementation of magnetite, the blood samples were incubated for 72 h at 37°C. The addition o mitomycin C (MMC; C15H18N4O5; Sigma®, St, Louis/MO, USA, at 10^{-7}M) served as a positive control (control$^+$). Each individual whole blood culture without magnetite served as a negative control (control$^-$) group.

Cytotoxicity tests

MTT assay

Cytotoxic testing was performed by measuring cell viability using the MTT colorimetric assay. Cytotoxicity was assessed by measuring the formation of formazan from MTT. Blood cells were incubated with 0.7 mg/ml MTT for 30 min at 37°C for 24 h. After washing with PBS, the blue formazan was extracted from cells with isopropanol/formic acid (95:5). Absorbance was recorded at 560 nm (Lewerenz et al., 2003). Cell viability was expressed in percentages of viable cells.

Lactate dehydrogenase assay

Lactate dehydrogenase (LDH) activity was measured in the culture medium after 24 h as an index of cytotoxicity, using an LDH kit (Bayer Diagnostics®, France) adapted to the auto analyzer (ADVIA 1650, USA). Enzyme activity was expressed as the extracellular LDH activity percentage of the total activity in the tubes.

Genotoxicity tests

Sister chromatid exchange (SCE) assay

With the aim of providing successive visualization of SCE, 5-bromo-2-deoxyuridine (Sigma®) was added at culture initiation. The cultures were incubated in complete darkness for 72 h at 37°C. Exactly 70 h and 30 min after starting the incubations, demecolcine (N-Deacetyl-N-methyl colchicine, Sigma®) was added to the cultures. After hypotonic treatment (0.075 M KCl), followed by three repetitive cycles of fixation in methanol/acetic acid solution (3:1, v/v), centrifugation and resuspension, the cell suspension was dropped onto chilled, grease-free microscopic slides, air-dried, aged for 3 days, and then differentially stained for the inspection of the SCE rate according to fluorescence-plus-Giemsa (FPG) procedure.

Micronucleus (MN) assay

The MN assay was performed by adding cytochalasin B (Sigma®) after 44 h of culture. At the end of the 72-h incubation period, lymphocytes were fixed with ice-cold methanol/acetic acid (1:1, v/v). The fixed cells were put directly on slides using a cytospin, and stained with Giemsa solution. All slides were coded before scoring. The criteria for scoring MN were as described by Fenech (1993). At least 1000 binucleated lymphocytes were examined per concentration for the presence of one, two or more MN by one observer.

Chromosome aberration (CA) assay

Two h prior to harvesting, 0.1 mL of colchicine (0.2 mg/mL, Sigma®) was added to the culture flask. Hypotonic treatment and fixation were performed. To prepare slides, 3-5 drops of the fixed cell suspension were dropped onto a clean slide and air-dried. The slides were stained in 3% Giemsa solution in phosphate buffer (pH 6.8) for 15 min. For each treatment, 30 well-spread metaphases were analyzed to detect the presence of CA. Criteria to classify the different types of aberrations (chromatid or chromosome gap and chromatid or chromosome break) were in accordance with the recommendation of Environmental Health Criteria (EHC) 46 for environmental monitoring of human populations (IPCS, 1985).

Total antioxidant capacity (TAC) and total oxidant status (TOS) assays

The TAC test measures the antioxidant capacity of all antioxidants in a biological sample and not only the antioxidant capacity of a single compound. In this approach, antioxidants in the sample reduce the dark blue-green-colored ABTS radical to a colorless reduced ABTS form. The change of absorbance at 660 nm is related to the total antioxidant level of the sample or compound. The assay is calibrated with a stable antioxidant standard solution or Trolox Equivalent, a vitamin E analog. In the TOS assay, oxidants present in the sample oxidize the ferrous ion-chelator complex to ferric ion. Enhancer molecules that are abundantly present in the reaction medium prolong the oxidation reaction. The ferric ion makes a colored complex with chromogen in an acidic medium. The color intensity, which can be measured spectrophotometrically, is related to the total amount of oxidant molecules present in the sample. The TOS assay is calibrated with hydrogen peroxide and the results are expressed in terms of μmol H_2O_2 equiv./L). The TAC and TOS assays were carried out in culture medium by commercially available kits (Rel Assay Diagnostics®, Turkey) in plasma samples obtained from blood cultures.

Nucleic acid oxidation

DNA oxidation was determined by measuring the amount of 8-OH-dG adducts. DNA was digested by incubation with DNase I, endonuclease and alkaline phosphatase. The amount of 8-OH-dG was measured by high-performance liquid chromatography (HPLC) with electrochemical detection as described previously (Floyd et al., 1986).

Statistics

The experimental data were analyzed using one-way analysis of variance (ANOVA) and Fischer's least significant difference (LSD) tests to determine whether any treatment significantly differed from the controls or each other. Results are presented as means±SD values and levels of 0.05 were regarded as statistically significant.

RESULTS

Fig. 3 shows the results of cytotoxicity measured by the MTT assay. When assayed *in vitro* on whole human blood cells using the MTT assay, the percentage of viable cells for the MMC-treated cells ($control^+$) was 1.8-fold lower than that for the $control^-$ cells. Likewise, the higher concentrations of magnetite (100, 150, 300, 500 and 1000 mg/L) caused significant ($p<0.05$) decreases in the cell viability. However, the human blood cells exposed to doses lower than 100 mg/L of magnetite did not show any significant change in cell viability

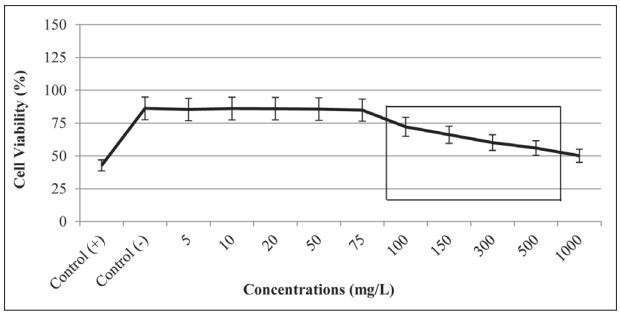

Fig. 3. Effect of magnetite on the viability of human whole blood cells as measured by the MTT assay after 24 h of incubation. Values inside the rectangle are statistically different from the corresponding control (-) (n=6).

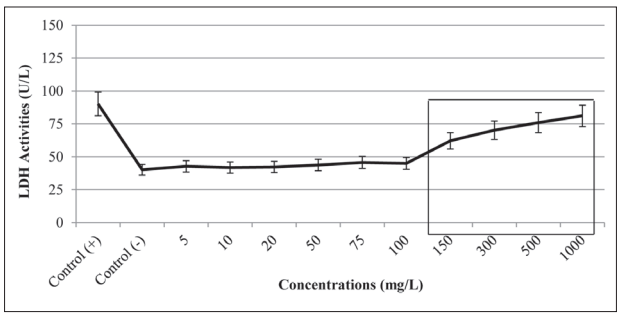

Fig. 4. LDH activities on cultured human blood cells treated with different concentrations of magnetite for 24 h. Values inside the rectangle are statistically different from the corresponding control (-) (n=6). LDH: lactate dehydrogenase.

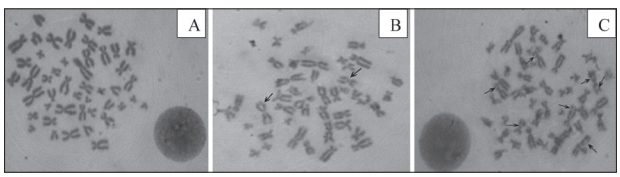

Fig. 5. Chromosome aberration images of human lymphocytes treated with different concentrations of magnetite (A: Control (-); B: 20 mg/L magnetite; C: 50 mg/L magnetite).

during 24 h as determined by the MTT assay, and no cytotoxicity was observed for control$^-$ cells. MMC-induced hematological damage was clearly evidenced by 7-fold increases in the activity of LDH compared with observations of the negative controls (Fig. 4). Although LDH was not affected by low doses of magnetite alone, increases in LDH levels reached statistical significances at 150, 300, 500 and 1000 mg/L.

The results of the sister SCE, MN and CA assays in human lymphocyte cells after 72-h magnetite treatment are presented in Table 1. SCE (at 20, 50 and 75 mg/L), MN (at 50 and 75 mg/L) and CA (at 20, 50 and 75 mg/L) analyses showed statistically significant differences ($p<0.05$) between control$^-$ and magnetite-applied cultures (Fig. 5). However, high concentrations of magnetite (higher than 100 mg/L) caused sterility of the cultures due to its cytotoxic activity after 72 h.

Table 1. The rates of SCEs and frequencies of CAs and MNs in human lymphocytes treated with different concentrations of magnetite *in vitro*.

	Genotoxicity assays		
Treatments	**CAs/cell**	**MN/1000 cells**	**SCEs/cell**
Control$^-$		4.5±1.8^a	6.5±0.9^a
Control$^+$	2.29±0.36^b	14.3±3.6^b	9.8±1.7^b
5 mg/L	0.19±0.02^a	4.6±1.2^a	6.3±1.0^a
10 mg/L	0.22±0.04^a	4.9±1.4^a	5.9±1.3^a
20 mg/L	1.26±0.17^b	5.2±1.3^a	7.9±1.4^b
50 mg/L	1.48±0.15^b	7.5±2.1^b	8.1±1.4^b
75 mg/L	1.54±0.16^b	8.1±2.3^b	8.3±1.5^b
100 mg/L	-	-	-
150 mg/L	-	-	-
300 mg/L	-	-	-
500 mg/L	-	-	-
1000 mg/L	-	-	-

Values are means±standard deviation (n=6), means in the same column followed by different letter are significantly different.

Figs 6 and 7 show the effects of magnetite concentrations on oxidant status in human whole blood cultures determined by TAC and TOS analysis. The TAC value decreased (Fig. 6) with the addition of MMC, while the TOS value increased (Fig. 7). In contrast, 20 and 50 mg/L concentrations of magnetite did not lead to any alterations in TAC levels, while 10 and 5 mg/L of magnetite caused significant increases in TAC levels in cultured human blood cells as compared to the control value (Fig. 6). Also, magnetite caused significant decreases in TAC levels at high concentrations (75, 100, 150, 300, 500 and 1000 mg/L). On the other hand, the TOS levels did not change at 5- and 10-mg/L doses of magnetite in cultured human blood cells (Fig. 7). However, magnetite increased the TOS levels in cultured human blood cells at doses higher than 10 mg/L. Thus, magnetite had dose-dependent effects on oxidative damage in human blood cells.

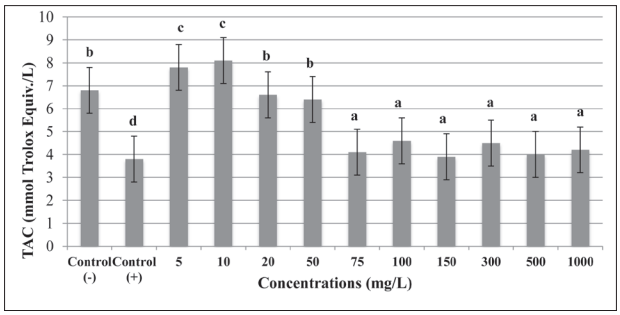

Fig. 6. TAC levels in human blood cells maintained for 2 h in the presence of magnetite NPs *in vitro*. Values are means±standard deviation (n=6), means in the same column followed by different letter are significantly different at the ($p<0.05$) level.

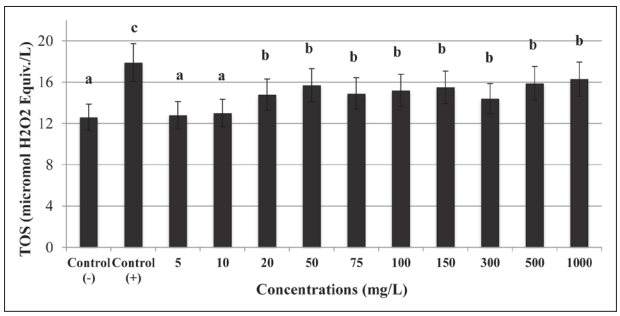

Fig. 7. TOS levels in human blood cells maintained for 2 h in the presence of magnetite NPs *in vitro*. Abbreviations are as in Fig. 6.

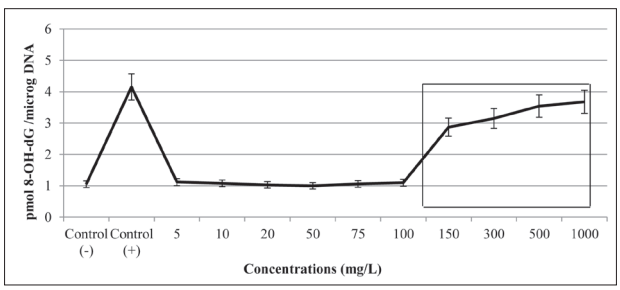

Fig. 8. 8-OH-dG adducts in cultured human blood cells maintained for 72 h in the presence of magnetite. Values inside the rectangle are statistically different from the corresponding control (-) (n=6). Positive control: mitomycin C (10^{-7} M).

The status of 8-OH-dG in cultured human blood cells of control and experimental groups is presented in Fig. 8. The levels of 8-OH-dG, a sensitive marker of oxidative DNA damage, were quantified. We observed that MMC at 10^{-7} M significantly increased the concentration of 8-OH-dG in human blood cultures after 72 h. Likewise 8-OH-dG levels also increased in the blood cells treated with 150, 300, 500 and 100 mg/L of magnetite.

DISCUSSION

The use of magnetite NPs in consumer products and biomedical applications is rapidly expanding. As a consequence, industry workers, consumers and the environment are anticipated to be increasingly exposed to magnetite NPs. As one of the potential routes of human exposure is by inhalation of particles, a human lymphocyte cells was used in the present study. We observed that magnetite at concentrations above 100 mg/L induced a decrease in the percentage of viable cells, while at concentrations above 150 mg/L, magnetite treatments led to a pronounced LDH release in cultured human blood cells as compared to control values. Our results are in agreement with a previous study (Yazdi et al., 2012), which reported that magnetite NPs demonstrated strong cytotoxicity towards 4T1 breast cancer cells. The possible cytotoxic effects of magnetite have been reported by various researchers (Ding et al., 2010; Könczöl et al., 2011).

Oxidative stress is an important mechanism of cytotoxic actions of NPs (Boczkowski and Hoet, 2010). The ability of NPs to generate reactive oxygen species (ROS) served to evaluate their toxic and cytotoxic effects (Xia et al., 2006; Foucaud et al., 2007; Meng et al., 2009). Various NPs can generate ROS production in experimental models (Limbach et al., 2007; AshaRani et al., 2009; Park and Park, 2009). This may be due to their unique physicochemical properties and direct and indirect effects of NPs on different organelles, as they enter cells via a "Trojan-horse" type mechanism. After their intracellular accumulation, NPs are stored in lysosomal vacuoles (Ortega et al., 2014). Mitochondria are the main sources of cellular ROS (Cash et al., 2007; Kowaltowski et al., 2009). Choi et al. (2009) reported that iron oxide NPs exhibited cytotoxicity by inducing ROS production and increasing LDH release in human lung epithelial cells (both normal and cancerous), cervical adenocarcinoma cells (HeLa) and osteosarcoma cells.

Magnetite usage in biological applications should be validated through biosafety marker tests *in vitro* and *in vivo*. A sensitive marker test of biosafety is the genotoxicity test which reveals DNA-damage (Rieznichenko et al., 2012). SCE, MN and CA assays are sensitive and reliable methods for studying DNA or chromosomal damage (Geyikoglu et al., 2005; Turkez, 2008; Turkez and Togar, 2010; Turkez et al., 2012a and 2012b). In this study, a significant increase in the SCE and CA frequencies of human lymphocyte cells was induced by magnetite exposure at concentrations of 20, 50, and 75 mg/L as compared to the negative control ($P<0.05$). In addition, 50- and 75-mg/L concentrations of magnetite increased MN frequency in lymphocytes. These findings suggest that magnetite has a potential risk of genotoxicity. Our results are consistent with those of Könczöl et al. (2011), who found that magnetite showed genotoxic effects using CA, MN and Comet assays in human alveolar epithelial-like type-II cells (A549). Ahamed et al. (2013) revealed that exposure to iron oxide NPs induced genotoxicity with increasing levels of DNA damage in skin epithelial and lung epithelial cells. Likewise, an *in vivo* study on Swiss mice using polyaspartic acid-coated magnetite demonstrated a time- and dose-dependent increase in MN frequency (Sadeghiani et al., 2005). Karlsson et al. (2009) reported that iron oxide NPs exhibited genotoxic action in human alveolar type II-like epithelial cells. In contrast to our findings, iron oxide did not lead to genotoxicity in Syrian hamster embryo cells, as was revealed by MN and comet assays (Guichard et al., 2012). Szalay et al. (2012) also found non-mutagenic activity of iron oxide NPs in *Salmonella typhimurium* (TA100, TA1535, TA98 and TA1537 strains) and *Escherichia coli* (WP2uvrA strain). 8-OH-dG is a major product of oxidative DNA damage and as such is regarded as a useful and relevant marker for cellular oxidative stress, particularly with respect to carcinogenesis (Breimer, 1990; Halliwell, 1999). In our investigation, it was determined that magnetite increased the 8-OH-dG concentration in cultured human blood cells at doses higher than 10 mg/L as compared to control values. Similar to our findings, previous studies have shown that Fe_3O_4 NPs elicited an increase in 8-OH-dG levels in human lung epithelial cells (Watanabe et al., 2013). Again, a recent study indicated that iron oxide NPs increased 8-OH-dG levels in liver and kidney tissues of mice (Ma et al., 2012).

Evaluation of the antioxidant activity of NPs was considered as their potentially important toxic property (Karunakaran et al., 2013). Milto et al. (2013) found that magnetite NPs exerted dose-dependent pro-oxidant and antioxidant properties in rat blood cells. In accordance to this *in vivo* finding, we observed that magnetite at concentrations of 5 and 10 mg/L led to increases in TAC levels in human blood cells. On the other hand, treatments with 5 and 10 mg/L of magnetite did not lead to any alterations in TOS levels, while at concentrations above 10 mg/L magnetite caused significant increases in TOS levels in cultured human blood cells as compared to control values. The prominent increase in TOS levels is accompanied by a significant decrease in TAC levels at concentrations of magnetite above 50 mg/L that could result in increased oxidative stress and consequently potential oxidative damage in human blood cells. Again, our findings supported the results of Buyukhatipoglu and Clyne (2011), which revealed that iron oxide NPs induced cellular ROS formation in endothelial cells. Another study reported that magnetite led to oxidative stress by reducing the level of intracellular reduced glutathione in the cells of bronco alveolar lavage fluid (Park et al., 2010).

CONCLUSION

The results obtained in the present study revealed that exposure to magnetite NPs produces oxidative DNA damage and is cytotoxic to human lymphocyte cells *in vitro* in a dose-dependent manner.

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Conflict of interest disclosure: The authors declare that there are no conflicts of interest.

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