Potential toxic effects of iron oxide nanoparticles in *in vivo* and *in vitro* experiments

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ABSTRACT: The aim of this study was to determine the potential toxic effects of iron(II,III)oxide nanoparticles (IONPs). In *in vivo* experiments, the toxic effects of IONPs were monitored in adult male Wistar rats by morphological methods after a single intratracheal instillation. For the control group 1 ml of physiological saline per animal was given, and the treatment group received the same volume of a suspension containing 1 and 5 mg kg^{-1} body weight IONPs. Lungs and internal organs underwent histopathological examination after 1, 3, 7, 14 and 30 days. The mutagenic effect of these nanoparticles was evaluated by the bacterial reverse mutation assay on *Salmonella typhimurium* TA98, TA100, TA1535 and TA1537 strains, and on *Escherichia coli* WP2*uvr*A strain, in the presence and absence of the mammalian metabolic activation system S9. The *in vitro* cytotoxic effect of IONPs was also examined in Vero cells after short-term (4 h) and long-term (24 h) exposure. There were no pathological changes in examined internal organs, except a very weak pulmonary fibrosis developing by the end of the first month in the treated rats. While *in vitro* MTT assay showed a moderate cytotoxic effect, IONPs proved to be devoid of mutagenic effect in the bacterial systems tested. The results may be a useful extension of our knowledge on the safety of magnetite nanoparticles in view of their possible medical applications, such as in hyperthermia and magnetic resonance imaging. Copyright © 2011 John Wiley & Sons, Ltd.

Keywords: iron(II,III) oxide nanoparticle (IONP); magnetite; lungs; MTT assay; bacterial reverse mutation assay

INTRODUCTION

In the past decade, nanomaterials have been increasingly applied for various purposes, including diagnostic and therapeutic ones, in technology and medicine, owing to their unique physicochemical properties (Dave and Gao, 2009; Jing *et al.*, 2010). Their small size and novel characteristics may, however, be responsible for adverse biological effects. Consequently, more recently the health and environmental safety of nanomaterials have drawn increasing attention, a distinct lack of information on impacts of engineered nanomaterials on human health and environment being highlighted (EC, 2004; RSRAE, 2004). Presently, the most important nanomaterials are simple metal oxides such as aluminium oxide (Al$_2$O$_3$), silica (SiO$_2$), zinc oxide (ZnO), copper oxide (CuO), titanium oxide (TiO$_2$) manganese oxide (MnO$_2$) and iron oxide (Fe$_3$O$_4$, Fe$_2$O$_3$; Balasubramanyam *et al.*, 2010; Fahmy and Cormier, 2009; Sárközi *et al.*, 2009; Oszlánczi *et al.*, 2010); yet the environmental and human health risks of these nanomaterials are largely unknown. Previous toxicological studies on nanomaterials were limited and conducted on TiO$_2$, CdO$_2$, C$_{60}$, and carbon nanotubes only (Pan *et al.*, 2010; Horváth *et al.*, 2011).

Iron(II,III)oxide nanoparticles (IONPs) have attracted much attention not only because of their magnetic properties but also owing to their great potential in several biomedical and *in vivo* clinical applications, such as drug delivery, magnetic resonance imaging (MRI), contrast enhancement, tissue repair and magnetic hyperthermia cancer treatment (Singh *et al.*, 2009; Naqvi *et al.*, 2010; Balasubramanyam *et al.*, 2010; Gupta *et al.*, 2007; Soenen *et al.* 2011; Sun *et al.*, 2007; Mahmoudi *et al.*, 2010). It has also been reported that IONPs can improve the efficiency of anticancer drugs and reverse multidrug resistance, and could hence be used as targeted drug carriers (Schroeder *et al.*, 1998; Lin *et al.*, 2007; Jarzyna *et al.*, 2009). The combination of IONPs with different chemotherapeutics may provide new clinical options also in the treatment of lymphoma (Jing *et al.*, 2010).

IONPs can be manufactured with surface modification to make them more biocompatible. They can be conjugated with suitable ligands to target specific receptors of cancer cells for developing targeted delivery systems (Naqvi *et al.*, 2010). Uncoated IONPs have very low solubility, which makes them suitable for various clinical applications (Singh *et al.*, 2009).

It is crucial to obtain further data on the general toxic effects and genotoxic potential of nanomaterials, given the potentially wide-ranging personal exposures, and the fact that, in clinical applications, individuals exposed to nanomaterials might be more vulnerable owing to their pre-existing medical conditions (Singh *et al.*, 2009).

A major aspect of harms caused by nanomaterials is mutagenicity (Singh *et al.*, 2009). Information on the mutagenicity so far is scanty, however, and it cannot be reliably predicted from the physical and chemical properties of the bulk materials and solutes that are used

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to make the nanoparticles (NPs; Kumar *et al.*, 2011). Generally, the toxic responses observed for nanoparticles *in vitro* (Mahmoudi *et al.*, 2010) may not be exactly reproduced *in vivo*. This may be due to the well-known principle of body homeostasis, counteracting harmful effects in *in vivo* assays so that few detectable changes are observed. The poor correlation between *in vivo* and *in vitro* toxicity outcomes may also be due to the ability of nanoparticles to change the cell medium during *in vitro* assays, for example by protein/ion adsorption and pH changes, caused in part by the surface activity of superparamagnetic iron oxide nanoparticle (SPIONs; Mahmoudi *et al.*, 2010).

In case of IONPs, *in vivo* application in imaging and drug delivery is very promising, but it is also important to know the safe upper limit of IONPs in such use (Gupta and Curtis, 2004; Sonvico *et al.*, 2005). There are a few reports available (Gupta and Curtis, 2004; Zhang *et al.*, 2002; Naqvi *et al.*, 2010), but more detailed studies are needed to evaluate the exact toxic effect of IONPs on cellular functions.

Despite these concerns, only limited studies have been actually conducted to assess the health effect of nanomaterials using *in vivo* and *in vitro* methods. The aim of this work was to advance the study of the general toxic, cytotoxic and mutagenic effects of magnetite nanoparticles using *in vivo* (general toxicology and histopathology) and *in vitro* (mutagenicity and cytotocicity tests) methods.

MATERIALS

Chemicals

Iron(II,III)oxide nanoparticles, dimethylsulfoxide (DMSO; purity > 99.5%) were obtained from Sigma-Aldrich Co. (Budapest, Hungary). The characteristics of IONP as reported by the manufacturer are: iron(II,III)oxide (Aldrich) nanopowder, spherical, <50 nm particle size (transmission electron microscopy), ≥98% trace metals basis, BET surface area >60 $m^2 g^{-1}$. The mutagens 2-aminoanthracene (2AA; purity 96%), sodium azide (SAZ; purity > 99.5%), methylmethane sulfonate (MMS; purity 99%), 9-aminoacridine (9AAC; purity 98%) and 4-nitro-1,2-fenilendiamin (NPDA; purity 98%) were obtained from Sigma–Aldrich Co. (Budapest, Hungary). SAZ, MMS and 9AAC were dissolved in deionized water, while NPDA and 2AA were dissolved in DMSO.

Cell Line and Culture

Vero cell line (C1008, ATCC no. CRL-1586TM) was obtained from the National Centre for Epidemiology (Budapest, Hungary). The Vero cells were cultured in RPMI 1640 medium (Sigma-Aldrich) containing 10% (v/v) heat-inactivated fetal bovine serum, 100 U ml^{-1} penicillin and 100 $\mu g\, ml^{-1}$ streptomycin at 37 °C in a humidified 5% CO_2 incubator, and passaged once every 2–3 days. Serum and antibiotics were obtained from Sigma-Aldrich Co. (Budapest, Hungary).

Medium and Rat Liver S9-based Metabolic Activation System

Nutrient broth no. 2 was obtained from Oxoid (Basingstoke, UK) and the bacteriological agar from Lab-Ka Ltd (Karcag, Hungary). Nutrient agar was prepared by adding 25 g Nutrient broth no. 2 and 12 g bacteriological agar to 1000 ml deionized water.

The S9 metabolic activator was prepared immediately before use, by adding: phosphate buffer (0.2 M) 5 ml; S9 fraction, 1 or 2 ml (10 or 20%); deionized water, 3 ml; $MgCl_2\, 6H_2O$ (Reanal, Hungary; 8 mM), 1.3 mg; KCl (Reanal; 33 mM), 24.6 mg; glucose-6-phosphate (Sigma; 5 mM), 14.1 mg; and NADP (Reanal; 4 mM), 3.7 mg. The S9 mixture was kept on ice during testing.

The S9 fraction [i.e. the liver postmitochondrial supernatant of rats treated with the mixture phenobarbital/β-naphthoflavone (PB/NF) to induce the hepatic microsomal enzymes] was stemmed from the Laboratory of Environmental Mutagenesis (National Institute of Environmental Health, Budapest). Protein concentrations of the S9 fractions were determined according to Lowry *et al.* (1951) using bovine serum albumin as standard.

Bacterial Strains

Salmonella typhimurium TA98 (hisD3052 rfa ΔuvrB pKM101), *S. typhimurium* TA100 (hisG56 rfa ΔuvrB pKM101), *S. typhimurium* TA1535 (hisG46 rfa ΔuvrB), *S. typhimurium* TA1537 (hisC3076 rfa ΔuvrB) and *Escherichia coli* WP2uvrA (trpE65 ΔuvrA) were supplied by Xenometrix AG (Allschwil, Switzerland). The strain genotypes were confirmed by testing the presence of specific genetic markers and phenotypes in preliminary strain check assays. Permanent cultures were afterwards prepared and frozen.

The working cultures used in each experiment were obtained from the frozen permanent cultures after incubation overnight (16 h) at 37 °C to reach a concentration of approximately 1×10^9 bacteria ml^{-1}. In each experiment the number of viable cells for each strain was defined according to OECD guidelines for testing of chemicals (OECD, 1997).

METHODS

In Vivo Approaches

Animals and treatment

Adult male Wistar rats obtained from the breeding centre of the University of Szeged were used (8 weeks old, 250–270 g body weight at start; see Fig. 1). The rats were housed in clean polypropylene cages and maintained in an air-conditioned conventional animal house at 22 ± 2 °C, 50–70% relative humidity and 12 h light/dark cycle (light on at 06:00). The animals were provided with commercial rat pellet (CRLT/N; Sindbad Ltd, Gödöllő, Hungary) and tap water *ad libitum*. After one week acclimatization, the rats were randomly divided into four groups: untreated control, control and low-dose and high-dose IONPs. Each group contained 30 animals each at the start. The rats were treated once during the experiment, and six of the 30 rats per group were sacrificed after 1, 3, 7, 14 and 30 days, respectively.

The average particle size and shape of the nanoparticles were checked by transmission electron microscopy (TEM; Fig. 2). For administration, the IONPs were suspended in physiological saline and instilled into the rat's trachea (for details of the technique, see Sárközi *et al.*, 2009). An untreated control (UnC, neither anesthesia nor intratracheal instillation) and a vehicle control group (Con, anesthetized and vehicle treated) were used. Doses, group coding and treatment scheme are shown in Table 1. The instilled volume was 1 ml kg^{-1} body weight. Before and during instillation, the nanosuspension was repeatedly sonicated (Elmasonic E15H, Germany) to prevent aggregation and sedimentation.
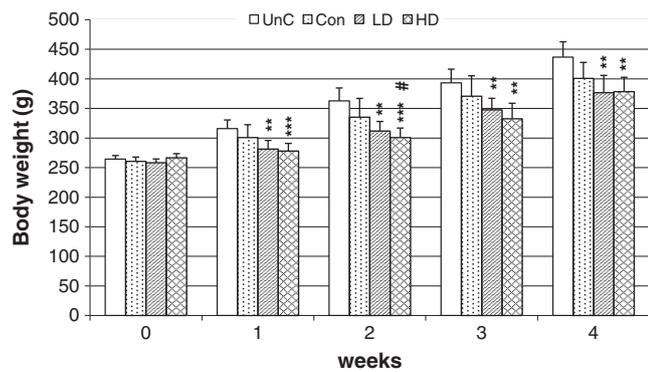
Figure 1. Body weight gain during the experiment of single intratracheal administration of iron(II,III)oxide nanoparticles. Abscissa, weeks after treatment; ordinate, body weight (mean $\pm$ SD, $n = 6$ per group). Descriptions of group codes are shown in Table 1. Significance: ** *** $P < 0.01$, 0.001 vs untreated control; # $P < 0.05$ vs vehicle treated control.
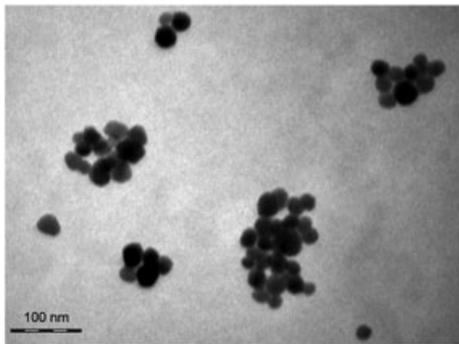
Figure 2. Transmission electron microscopy image of size and morphology of iron(II,III)oxide nanoparticles. Scale bar: 100 nm.

General toxicology and histopathological examination

The rats' body weight was recorded before NP administration, and then every 2 days and once more on the day of sacrifice. In halothane narcosis, the animal was exsanguinated by cutting the abdominal aorta and was dissected. The organ weights of the brain, liver, lungs, heart, kidneys, spleen, thymus and adrenals were measured. From these data, relative weights were calculated by relating organ weights to brain weight. Brain weight was used as reference (Schärer, 1977), because (in contrast to body weight) it was minimally affected by the treatment. The brain weights after 30 days were as follows: Con,

2.093 ± 0.063 g; low-dose (LD), 2.049 ± 0.045 g; and high-dose (HD), 2.111 ± 0.100 g.

Small tissue samples from the mentioned organs were fixed in 8% neutral buffered formalin, embedded in paraffin, sectioned at 5–6 µm thick, and stained with hematoxylin–eosin using standard histopathological techniques. The sections were examined by light microscopy.

In Vitro Techniques

MTT assay

Vero cells were grown in 96-well plates (3000 cells per well) until subconfluent. IONPs were then added to the cells at 78, 156, 313, 625, 1250, 2500, 5000 and 10 000 µg ml^{-1} concentration, and incubated for 4 and 24 h. After incubation, the medium was discarded and 90 µl fresh medium per well was added to the cells after washing with sterile phosphate-buffered saline. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide; Sigma] and 10 µl reagent (5 mg ml^{-1} stock) were then added per well and the plate was incubated for 4 or 24 h. After incubation, the medium was discarded from the wells and 200 µl methanol (Merck, Hungary) was added to solubilize the formazan crystals formed.

Reduction of MTT was quantified by optical densities (OD) at a measurement wavelength of 570 nm and a reference wavelength of 630 nm, using an absorbance microplate reader (EL$_x$808^{TM}; Bio-Tek, USA). Percentage viability of the cells was calculated as the ratio of mean absorbance of triplicate readings of sample wells (I_{sample}) compared with the mean absorbance of control wells ($I_{control}$): cell viability (%) = (I_{sample}/$I_{control}$) × 100.

Bacterial reverse mutation assay (Ames test)

In a preliminary experiment we assessed the solubility of IONPs in the final treatment mixture to find the highest concentration to be used in the following assays. Insolubility was defined as the formation of a precipitate of the substance in the final mixture under the test conditions and evident to unaided eye (OECD, 1997). IONPs gave a precipitate at concentrations higher than 100 mg ml^{-1} (corresponding to 5 mg per plate), thus this concentration was selected as the maximum one to be tested. Starting from 5 mg per plate, serial dilutions were prepared using a dilution factor of about 1:3.

In each experiment DMSO was used as a negative control and various diagnostic mutagens were included as positive controls: SAZ (1–2 µg per plate) for *S. typhimurium* tester strains TA1535 and TA100 without S9 mixture; NPDA (4 µg per plate)

Table 1. Treatment groups and the corresponding doses

Group	Number of animals	Code	Treatment and dose	Time of sacrifice
Untreated control	30	UnC	—	1, 3, 7, 14 and 30 days after single
Vehicle control	30	Con	0.9% physiology saline,1 ml kg^{-1} body weight	intratracheal instillation in all groups[a]
Low dose	30	LD	Fe(II,III)O nanosuspension,1 mg kg^{-1} body weight;1 ml kg^{-1} body weight	
High dose	30	HD	Fe(II,III)O nanosuspension,5 mg kg^{-1} body weight;1 ml kg^{-1} body weight	

[a]The groups started with 30 rats each; six rats per group were sacrificed after 1, 3, 7, 14 and 30 days.

for *S. typhimurium* tester strain TA98 without S9 mixture; 9AAC (1 μg per plate) for *S. typhimurium* tester strain TA1537 without S9 mixture; MMS (1 μl per plate) for *E. coli* tester strain WP2uvrA; 2AA (1–2 μg per plate) for all *S. typhimurium* tester strains; and 2AA (25 μg per plate) for *E. coli* WP2uvrA tester strain with S9 mixture.

The toxicity of the test materials was evaluated as reduction in the number of revertant colonies and as change in the auxotrophic background growth (background lawn) in comparison with control plates (Maron and Ames, 1983). A positive response in the test was defined as an increase (at least 2-fold above the control) in histidine- or tryptophan-independent revertant colonies in every strain (Ames *et al.*, 1975).

Statistical Analysis

All values were expressed as mean ± SD. From the general toxicological data, group means (± SD) were calculated. Results were tested for significance with one-way analysis of variance (ANOVA), and the MTT analyses were performed using the Student's *t*-test for unpaired data. *P*-Values < 0.05 were considered statistically significant. Ames test data were processed using standard statistical software COLONY (version 2.3, York Electronic Research, Huntington, York, UK) with the recommendation of UKEMS (UK Environmental Mutagen Society; Kirkland, 1994).

RESULTS

In Vivo Experiment: General Toxicity and Histopathology

Intratracheal instillation of IONPs (LD and HD) caused a significantly slowed body weight gain compared with UnC and Con groups from the first week onwards. UnC rats had normal weight gain during the treatment period and the weight gain in the Con group was similarly constant, although somewhat slower. Body weight gain was significantly reduced throughout the whole post-administration period in both treated groups (i.e. LD and HD; Fig. 1).

Among the relative organ weights (Table 2), the weight of the lungs decreased significantly with increasing dose and time (from the day 7 in HD, and day 14 in LD). It is noteworthy that physiological saline instillation (group Con) alone had minimal effect on the lung weight. By the first week, a significant decrease of the liver and kidney relative weight had also developed, but disappeared later. Apart from the lungs, no significant changes could be observed in other organs by the fourth week.

Pathological examination revealed no abnormalities in the exposed rats' organs (regional lymph nodes and internal organs compared with UnC or Con) except in the lungs. In the treated rats' lungs, after a focal, interstitial inflammation, a weak pulmonary fibrosis developed by the end of the first month. The degree of pulmonary fibrosis was higher in the HD than in the LD rats (Fig. 3).

In Vitro Experiment: MTT Results

The results of the MTT assay demonstrated that IONPs induced time- and concentration-dependent cytotoxicity in 24 h. Cell viability changed from 12.8% to approximately 110% with decreasing concentration of IONPs (10 000, 5000, 2500, 1250, 625, 313, 156 and 78 μg ml^{-1}). With only 4 h incubation at the same concentrations of NPs, the cell viability was similar to negative control (RPMI 1640 medium; Fig. 4). These results showed that IONPs had moderate cytotoxic effect.

Table 2. Relative organ weights (mean ± SD, *n* = 6 per group;[a] related to brain weights) in the control and treated rats 7, 14 and 30 days after single exposure to iron(II,III)oxide nanoparticles

Groups	UnC	Con	LD	HD
Relative organ weights, 7 days				
Lung	1.394 ± 0.150	1.141 ± 0.168	1.149 ± 0.160	1.116 ± 0.145*
Liver	8.841 ± 0.555	7.773 ± 0.807	7.339 ± 0.519**	6.408 ± 0.709**,†
Kidney	1.682 ± 0.081	1,408 ± 0,138*	1.334 ± 0.079***	1.205 ± 0.134***,†
Heart	0.476 ± 0.013	0.549 ± 0.086	0.552 ± 0.079	0.464 ± 0.051
Spleen	0.553 ± 0.047	0.439 ± 0.051*	0.445 ± 0.081	0.427 ± 0.090
Thymus	0.288 ± 0.049	0.299 ± 0.048	0.282 ± 0.029	0.323 ± 0.090
Relative organ weights, 14 days				
Lung	1.498 ± 0.135	1,214 ± 0,184	1.188 ± 0.054**	1.105 ± 0.119**
Liver	7.911 ± 0.679	7.476 ± 0.593	7.355 ± 0.497	7.876 ± 0.866
Kidney	1.444 ± 0.040	1.356 ± 0.094	1.467 ± 0.040†	1.350 ± 0.108
Heart	0.517 ± 0.041	0.519 ± 0.030	0.509 ± 0.015	0.535 ± 0.047
Spleen	0.442 ± 0.051	0.439 ± 0.057	0.424 ± 0.051	0.429 ± 0.021
Thymus	0.288 ± 0.044	0.300 ± 0.086	0.264 ± 0.021	0.278 ± 0.048
Relative organ weights, 30 days				
Lung	1.322 ± 0.109	1,113 ± 0,148	1.066 ± 0.124*	1.140 ± 0.119
Liver	7.875 ± 0.662	7.098 ± 0.689	7.000 ± 0.586	7.404 ± 0.626
Kidney	1.489 ± 0.090	1.329 ± 0.161	1.238 ± 0.144*	1.463 ± 0.123
Heart	0.527 ± 0.049	0.515 ± 0.019	0.481 ± 0.075	0.543 ± 0.028
Spleen	0.456 ± 0.049	0.412 ± 0.088	0.353 ± 0.042*	0.386 ± 0.052
Thymus	0.286 ± 0.053	0.250 ± 0.076	0.254 ± 0.057	0.222 ± 0.048

Significance: *, **, *** *P* < 0.05, 0.01, 0.001 vs UnC; † *P* < 0.05 vs Con. For group codes, see Table 1.
[a] From the 30 animals per treatment group six were sacrificed after 7, 14 and 30 days.
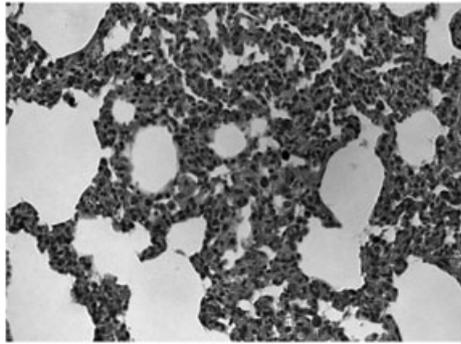
Figure 3. Lung tissue response in a rat exposed to high-dose iron(II,III) oxide nanoparticles. The lung displays abnormal architecture compared with untreated and vehicle controls 30 days after instillation of nanoparticles (hematoxylin–eosin; magnification $\times$ 280).
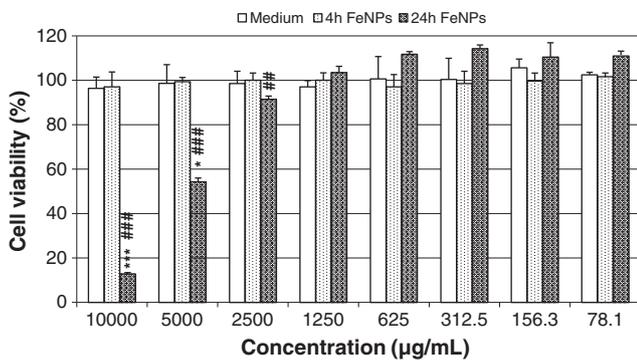
Figure 4. The effects of iron(II,III)oxide nanoparticles on cell viability of Vero cells as determined by MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-tetrazolium bromide] assay (mean $\pm$ SD). Significance: *, *** $P < 0.05$, 0.001 vs medium (as negative control); ##, ### $P < 0.01$, 0.001 vs 4 h iron(II, III) oxide nanoparticles.

In Vitro Experiment: Ames Test

A total of seven concentrations of IONPs (6.9–5000 μg per plate) were tested. The average number of revertant colonies was similar in the IONP-treated groups and the negative control. None of the revertant rates was greater than or equal to 2-fold the negative controls, and no concentration-dependent increase was observed. The results were identical with and without metabolic activation (Tables 3 and 4). In the negative control group of each tester strain, the average number of revertant colonies was within the range of the historical control data of our laboratory. The positive controls showed significant mutagenicity. These results demonstrated that IONPs are not mutagenic to the bacterial strains *S. typhimurium* TA100, TA1535, TA98, TA1537 and *E. coli* WP2uvrA.

DISCUSSION

The results presented provide a mixed toxicological picture of the tested IONPs. Marked general toxicity and organ toxicity (lungs) was seen *in vivo*, but only moderate mammalian cell toxicity and no mutagenicity *in vitro*.

Although magnetic (predominantly magnetite, Fe_3O_4, and maghemite, γ-Fe_2O_3) nanoparticles are the only nanomaterials that have been approved for clinical use to date (Gould, 2006),

Table 3. The number of reverse mutants of *S. typhimurium* TA100, TA1535, TA98, TA1537 and *E. coli* WP2uvrA with the Fe_3O_4 nanoparticles at various doses with and without 10% S9 activation

| Dose (μg per plate) | Base-pair substitution type | | | | | | Frameshift type | | | |
| | TA100 | | TA1535 | | WP2uvrA | | TA98 | | TA1537 | |
	−S9 mix	+S9 mix	−S9 mix	+S9 mix	−S9 mix	+S9 mix	−S9 mix	+S9 mix	−S9 mix	+S9 mix
0	136.8 ± 19.1	181.2 ± 22.3	16.5 ± 3.4	15.7 ± 3.6	76.3 ± 12.2	90.7 ± 9.7	34.8 ± 4.2	52.7 ± 5.5	18.3 ± 0.8	26.8 ± 6.6
6.9	147.7 ± 21.2	186.3 ± 23.5	13.3 ± 4.0	15.3 ± 4.0	78.7 ± 7.8	83.0 ± 4.0	33.0 ± 7.8	44.7 ± 2.9	17.0 ± 3.6	23.3 ± 4.0
20.6	136.7 ± 19.4	173.7 ± 16.4	11.7 ± 5.5	14.7 ± 0.6	89.3 ± 4.0	98.0 ± 10.8	35.0 ± 5.3	45.3 ± 3.8	18.3 ± 2.5	26.3 ± 11.9
61.7	148.0 ± 37.5	184.7 ± 9.5	14.7 ± 3.5	14.7 ± 1.5	81.7 ± 5.0	95.0 ± 7.2	37.3 ± 14.2	52.7 ± 5.1	13.7 ± 3.8	23.7 ± 1.5
185.2	149.3 ± 2.9	196.7 ± 11.9	15.7 ± 2.1	12.3 ± 1.2	99.3 ± 5.9	90.0 ± 12.8	32.0 ± 12.2	53.7 ± 11.0	18.0 ± 4.0	19.7 ± 2.5
555.6	156.3 ± 6.8	174.0 ± 7.6	12.7 ± 1.5	12.0 ± 1.0	88.7 ± 7.8	98.7 ± 8.0	28.7 ± 2.9	48.0 ± 2.0	14.7 ± 4.5	31.3 ± 4.2
1666.7	138.7 ± 16.0	191.0 ± 8.2	15.3 ± 3.1	13.7 ± 2.9	86.7 ± 12.9	89.3 ± 9.9	33.7 ± 6.7	59.7 ± 4.5	20.0 ± 3.6	31.3 ± 4.0
5000	161.3 ± 22.9	172.7 ± 24.9	11.0 ± 3.0	17.0 ± 8.7	80.0 ± 15.6	91.3 ± 21.9	41.3 ± 3.2	53.0 ± 3.6	13.3 ± 3.2	20.7 ± 2.3
Positive[a]	1282.0 ± 78.3	2470.0 ± 102.8	752.7 ± 38.4	470.7 ± 15.0	654.0 ± 31.8	876.3 ± 22.8	458.3 ± 42.4	2086.7 ± 58.0	445.3 ± 26.1	580.7 ± 32.1

[a]Positive controls: sodium azide (1–2 μg per plate) for TA1535 and TA100 without S9 mixture; 4-nitro-1,2-fenilendiamin (4 μg per plate) for TA98 without S9 mixture; 9-aminoacridine (1 μg per plate) for TA1537 without S9 mixture; methylmethane sulfonate (1 μl per plate) for WP2uvrA without S9 mixture; and 2-aminoanthracene (25 μg per plate) *E. coli* tester strain with S9 mixture.

Table 4. The number of reverse mutants of *S. typhimurium* TA100, TA1535, TA98, TA1537 and *E. coli* WP2uvrA with the Fe$_3$O$_4$ NPs at various doses with and without 20% S9 activation

Dose (μg per plate)	Base-pair substitution type						Frameshift type			
	TA 100		TA1535		WP2uvrA		TA98		TA1537	
	−S9 mix	+S9 mix	−S9 mix	+S9 mix	−S9 mix	+S9 mix	−S9 mix	+S9 mix	−S9 mix	+S9 mix
0	153.5 ± 28.2	133.5 ± 20.3	15.2 ± 3.2	21.7 ± 5.5	89.5 ± 12.2	100.7 ± 11.2	42.3 ± 6.0	62.5 ± 5.1	16.5 ± 5.3	28.3 ± 5.7
6.9	167.0 ± 12.0	160.3 ± 17.4	11.3 ± 4.9	22.3 ± 2.5	77.3 ± 10.0	93.0 ± 3.6	33.3 ± 1.2	57.3 ± 8.4	18.7 ± 2.1	27.3 ± 4.6
20.6	168.3 ± 5.0	166.7 ± 11.2	13.3 ± 4.7	16.7 ± 4.5	89.7 ± 3.5	114.7 ± 8.3	47.7 ± 1.5	60.7 ± 7.2	18.7 ± 2.9	32.3 ± 1.2
61.7	172.0 ± 7.6	170.3 ± 5.7	15.0 ± 5.6	19.3 ± 0.6	97.7 ± 6.4	100.0 ± 8.5	41.3 ± 4.0	57.0 ± 4.4	16.0 ± 2.7	31.0 ± 2.7
185.2	167.3 ± 13.1	155.0 ± 13.1	15.3 ± 2.5	20.3 ± 2.1	97.3 ± 14.4	94.7 ± 6.0	44.0 ± 12.5	55.3 ± 5.7	17.0 ± 5.3	25.7 ± 8.1
555.6	169.0 ± 15.4	170.7 ± 24.7	15.7 ± 2.5	13.7 ± 3.5	93.7 ± 7.8	110.0 ± 7.8	37.0 ± 7.8	51.0 ± 9.6	11.0 ± 2.7	23.7 ± 4.2
1666.7	156.7 ± 10.1	162.7 ± 8.5	11.3 ± 2.9	23.7 ± 3.1	81.3 ± 4.0	122.0 ± 19.7	31.7 ± 4.7	51.3 ± 11.4	16.0 ± 5.6	41.7 ± 4.5
5000	147.7 ± 14.8	175.7 ± 7.6	11.7 ± 0.6	18.3 ± 3.5	92.0 ± 15.6	103.7 ± 10.1	33.3 ± 3.2	48.7 ± 13.0	16.0 ± 2.7	24.7 ± 7.2
Positive[a]	1204.3 ± 91.6	1390.7 ± 80.0	887.7 ± 23.2	188.0 ± 28.7	396.3 ± 15.7	479.7 ± 29.7	520.0 ± 12.0	1262.0 ± 94.0	1497.0 ± 82.5	140.0 ± 20.8

[a]Positive controls: sodium azide (1–2 μg per plate) for TA1535 and TA100 without S9 mixture; 4-nitro-1,2-fenilendiamin (4 μg per plate) for TA98 without S9 mixture; 9-aminoacridine (1 μg per plate) for TA1537 without S9 mixture; methylmethane sulfonate (1 μl per plate) for WP2uvrA as well as 2AA (1–2 μg per plate) for all *S. typhimurium* tester strains; and 2-aminoanthracene (25 μg per plate) *E. coli* tester strain with S9 mixture.

studies on *in vivo* effects of IONPs are still scarce, and there has been some discrepancy in the literature about the adequacy of *in vivo* vs *in vitro* methods for the toxicological assessment of NPs and the interpretation of the results. *In vitro* assays may seem preferable, being simpler, faster and devoid of ethical problems as opposed to *in vivo* studies (Sayes *et al.*, 2007); the correspondence between *in vitro* and *in vivo* results is, however, questionable (Mahmoudi *et al.*, 2010).

In the lungs of the treated rats, after interstitial inflammation a weak pulmonary fibrosis was observed. In the rat model, lung exposure to nanoparticles typically produced greater adverse inflammatory and fibrotic responses than larger-sized particles of similar or identical composition at equivalent doses/mass concentrations (Warheit *et al.*, 2008), and this holds true for respirable (as opposed to nano-sized) iron oxide dust too (Parkes, 1982). This fact and our histopathological results underline the importance and necessity of further *in vivo* toxicological experiments with IONPs, even if inhalation is a less likely method of application of IONPs to humans.

The effect on body weight and the transient changes in the liver and kidney of the LD and HD rats indicated systemic action, which is in line with the observation of the presence of IONPs in the lumen of alveolar capillaries in a previous, nearly identical experiment (Szalay *et al.*, 2008). The responsible mechanism is most probably oxidative stress, the negative effect of which on body weight has been described after toxic exposure (Aoki *et al.*, 2002) and in a chronic stress model (Rezin *et al.*, 2008).

The *in vitro* cytotoxicity test revealed a moderate time- and concentration-dependent, cytotoxic effect of IONPs. With SPIONs applied at a 500 μg ml^{-1} dose, the viability of murine macrophage cells (J774) was reduced from 75 to 60% in 6 h (Naqvi *et al.*, 2010). Our results, covering a wider, possibly more realistic, dose range and exposure time range, also indicated cytotoxicity. In contrast to that, Soenen *et al.* (2011) examined four different types of coated IONPs, using the lactate dehydrogenase assay on C17.2 cells, and found no acute toxicity. Similarly, Ying and Hwang (2010) also reported that modification of the surface coating could overcome the cytotoxicity of IONPs. The role of the NPs' chemical composition (possibly, of the dissolved components) was shown by, for example, Fahmy and Cormier (2009), who found that CuO but not Fe$_2$O$_3$ or SiO$_2$ NPs induced cytotoxicity in HEp-2.

Jing *et al.* (2010) showed that, on concomitant treatment with daunorubicin (or adriamycin) and IONPs, the proliferation of Raji cells was markedly inhibited. Their results promote a potential clinical application for IONPs in combination with different chemotherapeutics against lymphoma – provided the toxic effect can be focused on the target cells.

Our Ames test data indicated no mutagenicity of the various concentrations of Fe$_3$O$_4$ NPs in the tester strains of *S. typhimurium* TA100, TA98, TA1535, TA1537 and *E. coli* WP2*uvr*A, with or without metabolic activation. This result is in line with a literature data on the mutagenic potential of metal oxides NPs. No mutagenic activity of Al$_2$O$_3$, Co$_3$O$_4$, TiO$_2$, and ZnO NPs to *S. typhimurium* TA97a, TA100, and *E. coli* WP2 trp uvrA was found, in both the absence and presence of S9 mixture (Pan *et al.*, 2010). Likewise, Al$_2$O$_3$ (30 and 40 nm) did not show mutagenicity in the tester strains of *S. typhimurium* TA100, TA1535, TA98, TA97a and TA102 (Balasubramanyam *et al.*, 2010). Kim *et al.* (2009) reported that Al$_2$O$_3$ NPs were not mutagenic on cultured mammalian cells examined with the mouse lymphoma assay (MLA). Yoshida *et al.* (2009) investigated ZnO NPs capped with tetramethylammonium hydroxide and found them nonmutagenic in the Ames test using

S. typhimurium strains TA98, TA100, TA1535 and TA1537 and *E. coli* WP2uvrA strains with and without S9 mixture. Others, however, found that certain NPs mutagenic. Iron-platinum (FePt) NPs tested were, with and without S9 mixture, weakly positive in the Ames test on *Salmonella* TA100 strain (Maenosono *et al.*, 2007). Kumar *et al.* (2011) showed the mutagenicity of TiO_2 NPs, in both the presence and absence of metabolic activation. They also demonstrated the uptake of ZnO and TiO_2 NPs in *S. typhimurium* (TA98 and TA1537) and the NPs' weak mutagenic potential, leading to frameshift mutations.

In conclusion, our findings suggested that IONPs do not cause mutagenicity as evaluated against various *S. typhimurium* and *E. coli* strains, but have moderate cytotoxic effect and cause interstitial inflammation in the lung after a very weak pulmonary fibrosis. Results of this study extend the knowledge on the safety of iron(II,III)oxide nanoparticles in view of their diagnostic and therapeutic applications.

Conflict of Interest

The authors declare that there are no conflicts of interest.

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