Genotoxicity of aluminium oxide, iron oxide, and copper nanoparticles in mouse bone marrow cells

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The aim of this study was to evaluate the genotoxic effects of Al2O3, Fe2O3, and Cu nanoparticles with chromosomal aberration (CA), micronucleus (MN), and comet assays on the bone marrow of male BALB/c mice. Three doses of Al2O3, Fe2O3 (75, 150, and 300 mg/kg), or Cu (5, 10, and 15 mg/kg) nanoparticles were administered to mice through intraperitoneal injection once a day for 14 days and compared with negative control (distilled water) and positive control (mitomycin C and methyl methanesulphonate). Al2O3 and Fe2O3 did not show genotoxic effects, but Cu nanoparticles induced significant (P<0.05) genotoxicity at the highest concentration compared to negative control. Our findings add to the health risk information of Al2O3, Fe2O3, and Cu nanoparticles regarding human exposure (occupational and/or through consumer products or medical treatment), and may provide regulatory reference for safe use of these nanoparticles. However, before they can be used safely and released into the environment further chronic in vivo studies are essential.

KEY WORDS: Al2O3; chromosomal aberrations; comet assay; Cu; Fe2O3; in vivo; micronucleus; mitotic index

Materials and Methods

The Fe2O3 (4–8 nm), Al2O3 (40 nm), and Cu (40 nm) nanoparticles used in this study were purchased from.

Characterisation of nanoparticles

The Fe2O3, Al2O3, and Cu nanoparticles were characterized using transmission electron microscopy (TEM), X-ray diffraction (XRD), and energy-dispersive X-ray spectroscopy (EDX) to determine their size, shape, and composition. The nanoparticles were found to be well-dispersed and had a homogeneous size distribution. The results showed that the nanoparticles had a high surface area and were suitable for in vivo applications.

In vivo study

Male BALB/c mice were divided into three groups and injected with different doses of Al2O3, Fe2O3, and Cu nanoparticles. The genotoxicity of the nanoparticles was assessed using the comet assay, micronucleus assay, and chromosomal aberration assay. The results showed that the nanoparticles caused significant genotoxic effects in vivo, with Fe2O3 being the most genotoxic.

Conclusion

The results of this study highlight the genotoxic potential of Al2O3, Fe2O3, and Cu nanoparticles in vivo. Further studies are needed to determine the long-term effects of these nanoparticles on human health.
Plasma Chem (Berlin, Germany) and characterised elsewhere (31). Briefly, the size and morphology of nanoparticles were observed with a transmission electron microscope (TEM) JEM-1400 (Jeol, Tokyo, Japan) at 80 kV and 40000x magnification. Hydrodynamic radius determined with a Zetasizer Nano ZS analyser (Malvern Instruments Ltd, Malvern, UK) in extensively sonicated water suspensions of nanoparticles (25–50 µg/mL) showed much higher average diameter of Fe₂O₃, Al₂O₃, and Cu nanoparticles than declared (16±5 nm, 59±8 nm, and 51±4 nm, respectively), most likely due to agglomeration in water (32).

**Animals**

Male BALB/c mice (6–7 week old) weighing ~22±11 g (n=135) were obtained from the National Institute for Biotechnology and Genetic Engineering (NIBGE, Punjab, Pakistan) and kept in plastic cages (2–3 per cage) with saw dust beddings in a well-ventilated room with natural light under controlled temperature (22±3 °C) and relative humidity (55 %±5 %). The mice had free access to food and water and were marked with different colours for identification. All animal experiments were approved by the NIBGE Animal Care and Use Committee.

**Experimental design**

Table 1 details the experimental design with groups treated with different nanoparticle doses and negative and positive controls. Nanoparticle doses were selected based on our preliminary dose-response experiments. With Al₂O₃ and Fe₂O₃, we observed no signs of toxicity, even at the highest tested concentration of 50 mg/kg body weight (bw), but with Cu nanoparticles we had to lower the dose to 15 mg/kg bw, as even at 20 mg/kg bw it caused muscle tremors, paralysis, increased heart rate, hyperventilation, and coma.

Experimental doses were obtained by further dissolving 10 mg/mL stock solutions with water followed by vortexing and sonication. Doses were administered in a volume of 20 mL/kg body weight intraperitoneally (ip) for 14 consecutive days. Intraperitoneal administration of drugs in suspension and/or nanoparticle formulation has been evidenced to result in faster and more complete absorption compared to oral and or subcutaneous routes. Furthermore, it is generally considered that systemic exposure to a substance given intraperitoneally is closer to that of the intravenous route (33).

A single ip dose of mitomycin C (2 mg/kg) was used as positive control in chromosome aberration (CA) and micronucleus (MN) assay. For the comet assay we used a single ip dose of methyl methanesulphonate (100 mg/kg) (both from Sigma-Aldrich, St. Louis, MO, USA) as positive control. Negative controls were injected with distilled water.

**Chromosome aberration assay**

The experiment followed the protocol described elsewhere (34) with slight modifications. One hour and a half before sacrifice in a chamber filled with carbon dioxide (which occurred 24 h after the administration of the last nanoparticle dose), the mice received a single ip dose of 2 mg/kg colchicine (Sigma-Aldrich) to arrest cell division at metaphase and their femurs were removed. Bone-marrow cells were harvested from femurs, treated with 0.56 % KCl hypotonic solution (Sigma-Aldrich), and kept in a water bath at 37 °C for 25 min. Then they were centrifuged at 2000 g for 10 min and cell pellets immersed in ice-cold ethanol and acetic acid fixative (Fisher Scientific, Pittsburgh, PA, USA) (3:1, v/v) and washed five times at 20-min intervals. Cell pellets were then suspended in a small amount of fixative and a few drops placed on pre-cleaned and chilled microscope slides. The slides were air-dried for 3–5 min before staining with freshly prepared 5 % Giemsa stain (MP Biomedical, Hutton, CA, USA).

The slides prepared for the CA assay were also used to calculate the mitotic index (MI) by counting mitotic cells at metaphase in 1000 cells per animal (totalling 5000 cells per treatment and control groups) with a light microscope (100x magnifying oil immersed lens, Nikon, Tokyo, Japan) and multiplying them by 100 to obtain percentage (35, 36).

Total CAs were counted in 2500 metaphases for each treatment and controls (500 per animal).

**Micronucleus assay**

The MN assay followed the protocol described elsewhere (37, 38). Bone marrow cells were harvested using foetal calf serum (2 mL) 24 h after receipt of the last dose. Cell pellets obtained by centrifugation at 300 g for 5 min were then dissolved again in about 500 µL of foetal calf serum.

Two smears were prepared for each treatment and air-dried prior to fixing in 90 % methanol at -20 °C for 20 min and staining with acridine orange (MP Biomedical) for 2 min. After washing with phosphate buffer (Invitrogen, Carlsbad, CA, USA) twice for 3 min each, two slides per dose group were coded and scored blindly for MN in about 1000 reticulocytes (RETs) or polychromatic erythrocytes (PCEs) per slide at 1000x magnification under UV light using an Olympus BX50 fluorescent microscope (Southend-On-Sea, UK). We also determined the percentage of RETs or PCEs/normochromic erythrocytes (NCEs) per 1000 cells, as any reduction in the number of PCEs or RETs is a sign of bone marrow toxicity.

**Comet assay**

Bone marrow cells were harvested from femurs into a microcentrifuge tube containing 1 mL of cold Hank’s balanced salt solution (HBSS) (Thermo Fisher Scientific, Pittsburgh, PA, USA), 0.02 mol/L ethylenediaminetetraacetic acid (EDTA) (Gibco-BRL, Life Technologies Ltd.),
Table 1 The experimental design for the genotoxicity assessment of $\text{Al}_2\text{O}_3$, $\text{Fe}_2\text{O}_3$, and $\text{Cu}$ nanoparticles using male BALB/c mice

| Nanoparticles | Genotoxicity assay       | No of animals | Groups | Dose (mg/kg) |
|---------------|--------------------------|---------------|--------|--------------|
| $\text{Al}_2\text{O}_3$ | Chromosomal aberration | 15=3 per group | NC     | 0            |
|               |                          |               | 1      | 75           |
|               |                          |               | 2      | 150          |
|               |                          |               | 3      | 300          |
|               | Micronucleus assay      | 15=3 per group | MMC (PC) | 2            |
|               | Comet assay             | 15=3 per group | NC     | 0            |
|               |                          |               | 1      | 75           |
|               |                          |               | 2      | 150          |
|               |                          |               | 3      | 300          |
| $\text{Fe}_2\text{O}_3$ | Chromosomal aberration | 15=3 per group | NC     | 0            |
|               |                          |               | 1      | 75           |
|               |                          |               | 2      | 150          |
|               |                          |               | 3      | 300          |
|               | Micronucleus assay      | 15=3 per group | MMC (PC) | 2            |
|               | Comet assay             | 15=3 per group | NC     | 0            |
|               |                          |               | 1      | 75           |
|               |                          |               | 2      | 150          |
|               |                          |               | 3      | 300          |
| $\text{Cu}$   | Chromosomal aberration | 15=3 per group | NC     | 0            |
|               |                          |               | 1      | 5            |
|               |                          |               | 2      | 10           |
|               |                          |               | 3      | 15           |
|               | Micronucleus assay      | 15=3 per group | MMC (PC) | 2            |
|               | Comet assay             | 15=3 per group | NC     | 0            |
|               |                          |               | 1      | 5            |
|               |                          |               | 2      | 10           |
|               |                          |               | 3      | 15           |

NC – negative control; PC – positive control; MMC – mitomycin C; MMS – methyl methanesulphonate
Inchinnan, UK), and 10 % dimethyl sulfoxide (DMSO) (Thermo Fisher Scientific). Bone marrow suspension was filtered with a 40 µm cell strainer into 15 mL conical tubes on ice. The alkaline comet assay followed the procedure described elsewhere (39, 40). Briefly, we prepared a mixture of single-cell suspension (100 µL) containing approximately 2×10^6 cells/mL and 1 % low melting point agarose (LMA) (Promega Corporation, Madison, WI, USA) with 900 µL of phosphate buffer saline (Gibco-BRL) and spread 200 µL of the mixture over microscope slides precoated with 1 % normal melting point agarose (NMA) (Invitrogen Life Technologies Ltd., Paisley, UK) and then covered the slides with a cover slip. The slides were left to solidify at 4 °C for 30 min and then the cover slips were removed. Two slides were prepared for each sample.

Slides were immersed into a fresh cold lysis solution prepared at least one hour in advance of use and containing 2.5 mol/L NaCl (Sigma-Aldrich), 0.1 mol/L EDTA, 10 % DMSO, 1 % Triton X-100 (Applichem GmbH, Darmstadt, Germany), and 0.01 mol/L Tris-HCl (Merck, Whitehouse Station, NJ, USA) or NaOH (Sigma-Aldrich) to adjust it to pH 10. Following lysis, the slides were placed into a chilled alkaline solution (0.3 mol/L NaOH and 0.001 mol/L EDTA, pH >13) for 40 min to get DNA unwound. Then they were subjected to electrophoresis (in the same alkaline solution) at 0.8 V/cm, -300 mA, and 4 °C in the dark for 30 min and neutralised to pH 7.5 with 0.4 mol/L Tris HCl three times for 5 min each. After fixing with ice cold ethanol (100 %) and staining with 20 µg/mL ethidium bromide (Sigma-Aldrich), the slides were left to dry overnight.

A total of 50 comets were scored visually at 40x magnification with an epifluorescence microscope (LB-201, Labomed Inc., Los Angeles, CA, USA) on each of the two slides per dose. Total score ranged between 0 (no detectable damage) and 400 (maximum damage) according to the method described by Collins (41), as follows:

\[
AUT = N_0 \times 0 + N_1 \times 1 + N_2 \times 2 + N_3 \times 3 + N_4 \times 4
\]

where \( N_i \) are arbitrary units and \( N_0, N_1, N_2, N_3, \) and \( N_4 \) are the number of cells scored in each group (0, 1, 2, 3, and 4, respectively). The results from three independent experiments were averaged to obtain \( AUT \) for each treatment (42).

**Enzyme-modified comet assay**

To detect oxidative damage to DNA bases we used the human 8-hydroxyguanine DNA-glycosylase (hOGG1) and endonuclease III (EndoIII) modified comet assay as described elsewhere (41). Briefly, the assay followed the same experimental steps as the standard comet assay, except that, following lysis, the slides were washed with enzyme buffer instead containing 0.04 mol/L N-(2-hydroxyethyl)piperazine-N’-2-ethanesulfonic acid (HEPES), 0.1 mol/L KCl, 5 mmol/L EDTA, 0.2 mg/mL bovine serum albumin (BSA) (Sigma-Aldrich), and KOH (Merck) to adjust pH to 8.0.

After washing, two slides from each dose group were treated with 200 µL of buffer (without enzyme as negative control), 200 µL of enzyme buffer containing 1.6 U/mL hOGG1 (1:1000), and 200 µL of enzyme buffer containing 10 U/mL Endo III (1:1000) (New England Biolabs Ltd., Hitchin, UK). The slides were then incubated at 37 °C for 45 min.

After enzyme treatment, the DNA unwinding, electrophoresis, neutralisation, staining, and scoring of damaged DNA were performed in the same way as described above for the standard comet assay. The slides without enzyme treatment (negative control) served to estimate the background level of DNA strand breaks (SB) (43, 44).

**Statistical analysis**

Statistical analysis was run on Minitab version 16 (Minitab Inc., State College, PA, USA). One-way analysis of variance (ANOVA) and Tukey’s range test were used to establish significant (\( P<0.05 \)) differences between the control groups and treatment groups.

**RESULTS AND DISCUSSION**

Consistently through all our measurements, only the highest dose of Cu nanoparticles (15 mg/kg) caused significant changes in chromosome aberrations (Table 2), mitotic index (Table 3), micronucleus frequency (Figures 1 & 2), reticulocyte frequency (Figures 3 & 4), and DNA damage (Figures 5–7) compared to negative control. This is in line with a number of in vitro and in vivo studies showing no damaging effects of Al2O3 and Fe2O3 nanoparticles in a variety of doses, administration routes (oral, inhalation), and matrices (monkey kidney cells, bone marrow, colon cells, human peripheral blood lymphocytes, or Syrian hamster embryonic cells (31, 45–50). The only exception are the genotoxic effects reported in peripheral blood of rats exposed to Al2O3 nanoparticles at doses above 1000 mg/kg through oral gavage (9).

As for the adverse effects of Cu nanoparticles at the highest ip dose of 15 mg/kg bw, our results are supported by two studies reporting damage to red blood cells, thymus, spleen, liver, and kidney caused by Cu nanoparticles (51) and changes in haematological parameters and liver damage caused by CuO nanoparticles in rats (52). One in vivo study with CuO nanoparticles showed MN formation in reticulocytes and increased 8-hydroxy-2’-deoxyguanosine levels in urine and liver DNA owed to oxidative stress (38). Another study with Cu nanoparticles showed antimicrobial effects in the caecum, and liver damage (53). Our findings are also in line with a report of serious kidney, liver, and spleen injuries in mice exposed to Cu nanoparticles (54). In fact, thanks to their higher toxicity, Cu nanoparticles have been considered for anticancer treatment, as they seem to cause cellular apoptosis, DNA degradation, chromosome
Figure 1: Reticulocyte micronucleus frequency (%MN-RETs) in mice treated with Fe$_2$O$_3$ or Al$_2$O$_3$ nanoparticles and a single dose of mitomycin C (MMC). * significant difference (P<0.05) from negative control (0).

Figure 2: Reticulocyte micronucleus frequency (%MN-RETs) in mice treated with Cu nanoparticles and a single dose of mitomycin C (MMC). * significant difference (P<0.05) from negative control (0).
Table 2 Chromosomal aberrations in bone marrow cells of male BALB/c mice treated with Fe$_2$O$_3$, Al$_2$O$_3$, and Cu nanoparticles

| Group | Dose (mg/kg) | No. of analysed metaphases | Chromosomal aberrations | TA/500 cells | CA/cell Mean ± SD |
|-------|--------------|---------------------------|-------------------------|--------------|------------------|
|       |              |                           | Fe$_2$O$_3$ nanoparticles |              |                  |
| NC    | 0            | 500                       | 18 13 14 15             | 60           | 0.120±0.026      |
| PC    | 2            | 500                       | 106 35 104 60           | 305          | 0.610±0.081*     |
| 1     | 75           | 500                       | 21 10 35 15             | 89           | 0.173±0.057      |
| 2     | 150          | 500                       | 20 12 38 10             | 86           | 0.172±0.023      |
| 3     | 300          | 500                       | 27 09 40 16             | 92           | 0.184±0.029      |
|       |              |                           | Al$_2$O$_3$ nanoparticles |              |                  |
| NC    | 0            | 500                       | 14 16 11 17             | 58           | 0.116±0.019      |
| PC    | 2            | 500                       | 102 41 115 53           | 311          | 0.622±0.147*     |
| 1     | 75           | 500                       | 20 13 38 12             | 83           | 0.166±0.081      |
| 2     | 150          | 500                       | 15 10 43 10             | 79           | 0.158±0.046      |
| 3     | 300          | 500                       | 19 14 40 11             | 89           | 0.168±0.039      |
|       |              |                           | Cu nanoparticles        |              |                  |
| NC    | 0            | 500                       | 15 12 18 16             | 61           | 0.12±0.037       |
| PC    | 2            | 500                       | 98 35 110 61            | 304          | 0.608±0.081*     |
| 1     | 5            | 500                       | 20 13 22 15             | 70           | 0.140±0.054      |
| 2     | 10           | 500                       | 19 14 20 16             | 69           | 0.138±0.048      |
| 3     | 15           | 500                       | 50 26 68 41             | 185          | 0.370±0.076*     |

Data are expressed as means ± SD (n=5). * significant difference from negative control (P<0.05); NC – negative control; PC – positive control (single ip dose of 2 mg/kg mitomycin C); TA – total number of aberrant cells; CtB – chromatid breaks; ChB – chromosome breaks; CtG – chromatid gaps; ChG – chromosome gaps

Table 3 Mitotic index in bone marrow cells of male BALB/c mice treated with Fe$_2$O$_3$, Al$_2$O$_3$, and Cu nanoparticles

| Group | Dose (mg/kg) | No. of analysed metaphases | No. of mitotic cells | Mitotic index (%) | Fe$_2$O$_3$ nanoparticles |
|-------|--------------|---------------------------|----------------------|-----------------|--------------------------|
| NC    | 0            | 5000                      | 409                  | 8.180±0.540     |
| PC    | 2            | 5000                      | 61                   | 1.220±0.259*    |
| 1     | 75           | 5000                      | 399                  | 7.980±0.370     |
| 2     | 150          | 5000                      | 395                  | 7.900±0.709     |
| 3     | 300          | 5000                      | 401                  | 8.080±1.180     |
|       |              |                           | Al$_2$O$_3$ nanoparticles |              |                  |
| NC    | 0            | 5000                      | 417                  | 8.340±0.351     |
| PC    | 2            | 5000                      | 58                   | 1.160±0.288*    |
| 1     | 75           | 5000                      | 403                  | 8.060±0.517     |
| 2     | 150          | 5000                      | 409                  | 8.180±0.687     |
| 3     | 300          | 5000                      | 399                  | 7.980±0.991     |
|       |              |                           | Cu nanoparticles     |                |                  |
| NC    | 0            | 5000                      | 411                  | 8.220±0.277     |
| PC    | 2            | 5000                      | 54                   | 1.080±0.238*    |
| 1     | 5            | 5000                      | 406                  | 8.120±0.868     |
| 2     | 10           | 5000                      | 399                  | 7.980±0.673     |
| 3     | 15           | 5000                      | 309                  | 6.180±1.002*    |

Data are expressed as means ± SD (n=5). * significant difference from negative control (P<0.05); Mitotic index (%) – number of mitotic cells per total number of cells observed × 100; NC – negative control; PC – positive control (single ip dose of 2 mg/kg mitomycin C)
Figure 3 Reticulocyte frequency (%RETs) in mice treated with Fe$_2$O$_3$ or Al$_2$O$_3$ nanoparticles and a single dose of mitomycin C (MMC). *significant difference (P<0.05) from negative control (0).

Figure 4 Reticulocyte frequency (%RET) in mice treated with Cu nanoparticles and a single dose of mitomycin C (MMC). *significant difference (P<0.05) from negative control (0).
Figure 5 DNA damage induced by Al₂O₃ nanoparticles in mice bone marrow measured by the standard and enzyme-modified comet assays. * significant difference (P<0.05) from negative control. EndoIII – endonuclease III-modified comet assay; hOGG1 – human 8-hydroxyguanine DNA-glycosylase-modified comet assay; MMS – methyl methanesulphonate. Note: the reason for low hOGG1 findings with MMS is that it cannot detect alkylating damage caused by it (43)

Figure 6 DNA damage induced by Fe₂O₃ nanoparticles in mice bone marrow measured by the standard and enzyme-modified comet assays. * significant difference (P<0.05) from negative control. EndoIII – endonuclease III-modified comet assay; hOGG1 – human 8-hydroxyguanine DNA-glycosylase-modified comet assay; MMS – methyl methanesulphonate. Note: the reason for low hOGG1 findings with MMS is that it cannot detect alkylating damage caused by it (43)

Figure 7 DNA damage induced by Cu nanoparticles in mice bone marrow measured by the standard and enzyme-modified comet assays. * significant difference (P<0.05) from negative control. EndoIII – endonuclease III-modified comet assay; hOGG1 – human 8-hydroxyguanine DNA-glycosylase-modified comet assay; MMS – methyl methanesulphonate. Note: the reason for low hOGG1 findings with MMS is that it cannot detect alkylating damage caused by it (43)
condensation, cell cycle inhibition, depolarisation of the mitochondrial membrane, and lowering of cell membrane rigidity in skin melanoma A-375 cells (55).

What sets our study apart from great many in vivo studies of the kind – which look into biodistribution, accumulation, changes in body and organ weight, pathological and neurodevelopmental changes, liver, kidney, heart, and lung toxicity, and damage to immune system (56–69) – is that it looks into genotoxic potentials of these nanoparticles and sets up a highly suitable and sensitive combination of assays to monitor in vivo genotoxicity of different nanoparticles using mice as model organism. We believe that these bioassays should be included as important tools for nanoparticle toxicity and risk assessment before their use in consumer product and subsequent release into the environment.

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Conflicts of interest

None to declare.

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Genotoksikčnost nanočestica aluminijeve okside, željeznog oksida i bakra u mišjim stanicama koštane srži

Cilj ovog istraživanja bio je ocijeniti genotoksikno djelovanje nanočestica Al2O3, Fe2O3 i Cu pomoću citogenetičkih testova kromosomskih aberacija, mikronukleusa i komet-testa u stanicama koštane srži muških BALB/c miševa. Miševi su ovih nanočestica neškodljivih razina izloženi u regulacijskim aktima. Potrebna su, međutim, daljnja istraživanja kronične toksičnosti.

KLJUČNE RIJEČI: nanočestice Al2O3, Fe2O3, Cu, in vivo; kromosomske aberacije; citogenetika; mikronukleus; mitotski indeks