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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 Al_2O_3, Fe_2O_3, and Cu nanoparticles with chromosomal aberration (CA), micronucleus (MN), and comet assays on the bone marrow of male BALB/c mice. Three doses of Al_2O_3, Fe_2O_3 (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). Al_2O_3 and Fe_2O_3 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 Al_2O_3, Fe_2O_3, 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: Al_2O_3; chromosomal aberrations; comet assay; Cu; Fe_2O_3; *in vivo*; micronucleus; mitotic index

Promising diagnostic and therapeutic applications of nanoparticles in medicine are owed to their different biokinetics and improved interactions with cells and sub cellular structures (1). Moreover, nanomaterials can pass biological barriers easily and exert their valuable or adverse effects (2).

However, their widespread use has also raised concern about their safety and potential risks for human health (3–5). This is particularly true for Al_2O_3, Fe_2O_3, and Cu nanoparticles, which have a wide range of industrial and medical applications and low production cost.

Al_2O_3 nanoparticles are used in biosensors, biofiltration, vaccination (as an adjuvant), drug delivery (cancer therapy), and are considered a promising anti-microbial agent and sorbent for heavy metals in waste water treatment (6–8), yet recent genotoxicity reports point to liver pathology (9, 10), carcinogenicity (11), inflammation (12), and cytotoxicity related to oxidative damage and loss of mitochondrial function (13).

Similar is true for Fe_2O_3 nanoparticles, used for targeted drug delivery, contrast-enhanced magnetic resonance imaging (MRI) (14–16), and thermal ablation therapy (17). A recent study (18) reported adverse effects on locomotor behaviour and spatial memory in mice receiving them intraperitoneally, most likely owed to nanoparticle accumulation, oxidative stress, DNA damage, and apoptosis. Another study in mice (19) has shown that Fe_2O_3 nanoparticles cause pathological changes in reproductive organs and the expression of heat shock gene through oxidative stress. Others have reported genetic damage, depletion in anti-enzymatic activity, and increase in lipid peroxidation in rats (20).

As for Cu nanoparticles, they have widely been used in the production of lubricants, polymers, ceramic pigments, metallic coating inks, and electronic devices (21–24). In medicine they can be used as a broad spectrum antimicrobial agent (25, 26). However, preliminary research of Cu nanoparticles shows their toxic effects *in vitro* and *in vivo*, such as cirrhosis and renal dysfunction in rats (27–30).

In fact, many researchers have studied the Fe_2O_3, Al_2O_3 and Cu nanoparticle genotoxicity *in vitro* in microorganisms and cell lines, but much is yet to learn about their genotoxicity *in vivo*. The aim of our study was therefore to add to scarce knowledge there is by studying the genotoxicity of Fe_2O_3, Al_2O_3 and Cu nanoparticles in mice bone marrow.

MATERIALS AND METHODS

Characterisation of nanoparticles

The Fe_2O_3 (4–8 nm), Al_2O_3 (40 nm), and Cu (40 nm) nanoparticles used in this study were purchased from

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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 Fe_2O_3, Al_2O_3, 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 Al_2O_3 and Fe_2O_3 nanoparticles 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, hypoventilation, 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 formulations 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 Biomedicals, 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 Biomedicals) 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/normochromatic 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 Al_2O_3, Fe_2O_3, and Cu nanoparticles using male BALB/c mice

Nanoparticles	Genotoxicity assay	No of animals	Groups	Dose (mg/kg)
Al_2O_3	Chromosomal aberration	15=3 per group	NC	0
			1	75
			2	150
			3	300
			MMC (PC)	2
	Micronucleus assay	15=3 per group	NC	0
			1	75
			2	150
			3	300
			MMC (PC)	2
	Comet assay	15=3 per group	NC	0
			1	75
			2	150
			3	300
			MMS (PC)	100
Fe_2O_3	Chromosomal aberration	15=3 per group	NC	0
			1	75
			2	150
			3	300
			MMC (PC)	2
	Micronucleus assay	15=3 per group	NC	0
			1	75
			2	150
			3	300
			MMC (PC)	2
	Comet assay	15=3 per group	NC	0
			1	75
			2	150
			3	300
			MMS (PC)	100
Cu	Chromosomal aberration	15=3 per group	NC	0
			1	5
			2	10
			3	15
			MMC (PC)	2
	Micronucleus assay	15=3 per group	NC	0
			1	5
			2	10
			3	15
			MMC (PC)	2
	Comet assay	15=3 per group	NC	0
			1	5
			2	10
			3	15
			MMS (PC)	100

NC – negative control; PC – positive control; MMC – mitomycin C; MMS – methyl methanesulphonate

Inchinnan, UK), and 10 % dimethyl sulphoxide (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:

$AU_T=N_0\times0+N_1\times1+N_2\times2+N_3\times3+N_4\times4$

where AU_T 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 AU_T 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-ethanesulphonic 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/mLhOGG1 (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 Al_2O_3 and Fe_2O_3 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 Al_2O_3 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

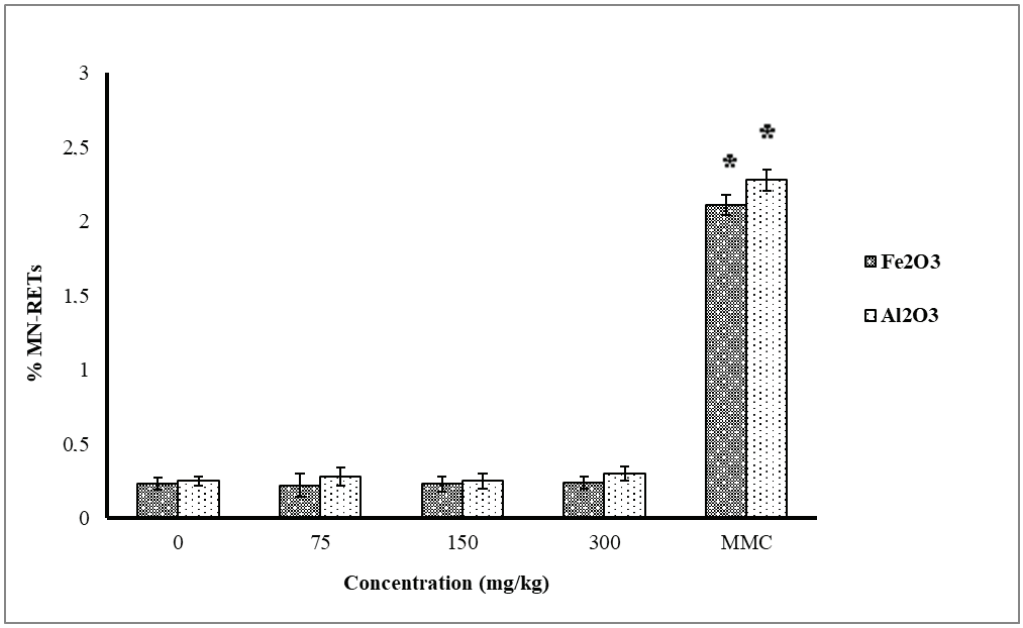

Figure 1 Reticulocyte micronucleus frequency (%MN-RETs) in mice treated with Fe_2O_3 or Al_2O_3 nanoparticles and a single dose of mitomycin C (MMC). * significant difference (P<0.05) from negative control (0)

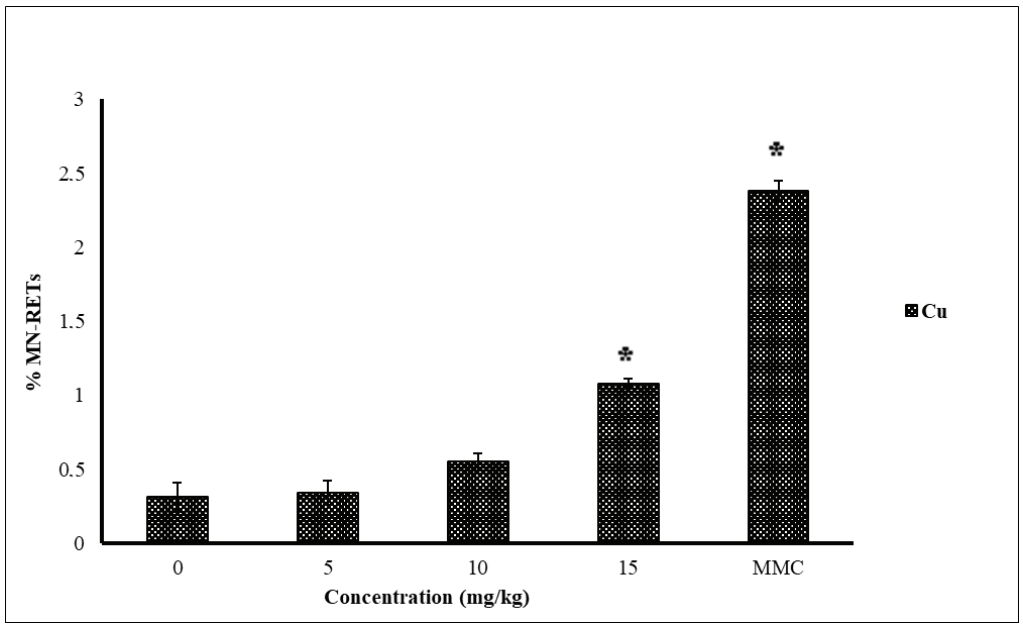

Figure 2 Reticulocyte micronucleus frequency (%MN-RETs) in mice treated with Cu nanoparticles and a single dose of mitomycin C (MMC). * significant difference (P<0.05) from negative control (0)

Table 2 Chromosomal aberrations in bone marrow cells of male BALB/c mice treated with Fe_2O_3, Al_2O_3 and Cu nanoparticles

Group	Dose (mg/kg)	No. of analysed metaphases	Chromosomal aberrations				TA/500 cells	CA/cell Mean ± SD
			CtB	ChB	CtG	ChG		
Fe_2O_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	0.178±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_2O_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_2O_3, Al_2O_3, and Cu nanoparticles

Group	Dose (mg/kg)	No. of analysed metaphases	No. of mitotic cells	Mitotic index (%)
Fe_2O_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_2O_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±0.802***

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)

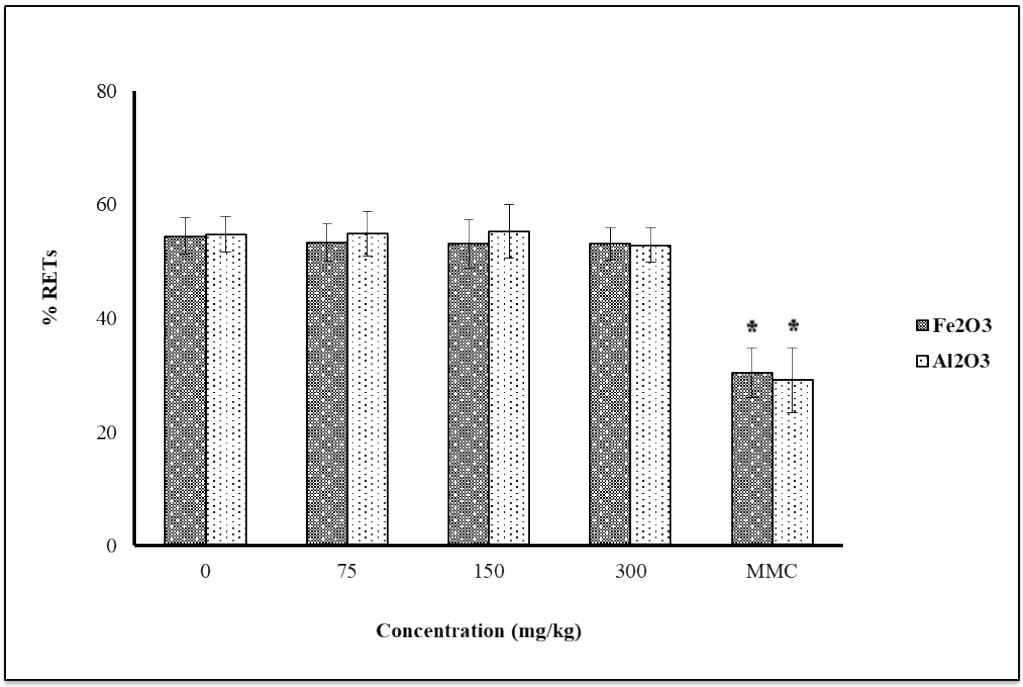

Figure 3 Reticulocyte frequency (%RETs) in mice treated with Fe_2O_3 or Al_2O_3 nanoparticles and a single dose of mitomycin C (MMC). *significant difference (P<0.05) from negative control (0)

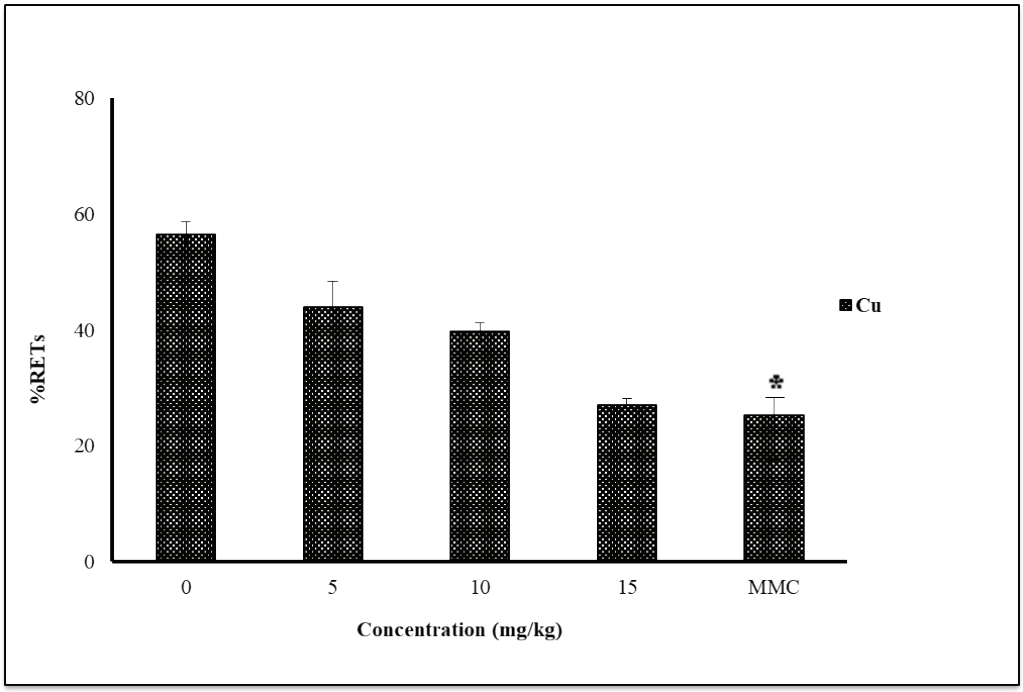

Figure 4 Reticulocyte frequency (%RET) in mice treated with Cu nanoparticles and a single dose of mitomycin C (MMC). *significant difference (P<0.05) from negative control (0)

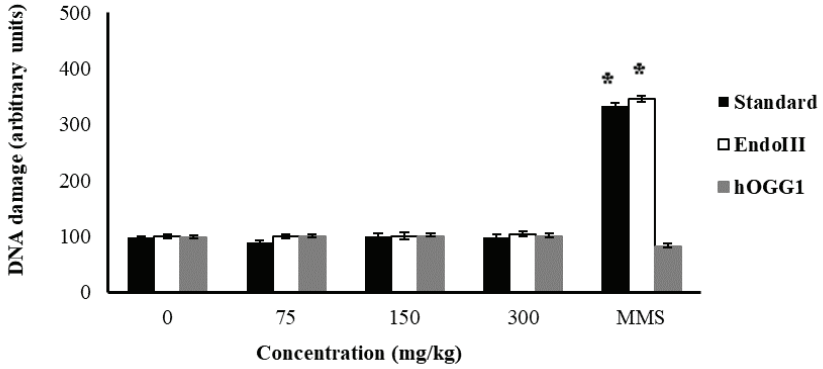

Figure 5 DNA damage induced by Al_2O_3 nanoparticles in mice bone marrow measured by the standard and enzyme-modified comet assays. * significant difference ($P<0.05$) from negative control. EndoIII – endonuclease III-modified comet assay; hOGG1 – human 8-hydroxyguanine DNA-glycosylase-modified comet assay; MMS – methyl methanesulphonate. Note: the reason for low hOGG1 findings with MMS is that it cannot detect alkylating damage caused by it (43)

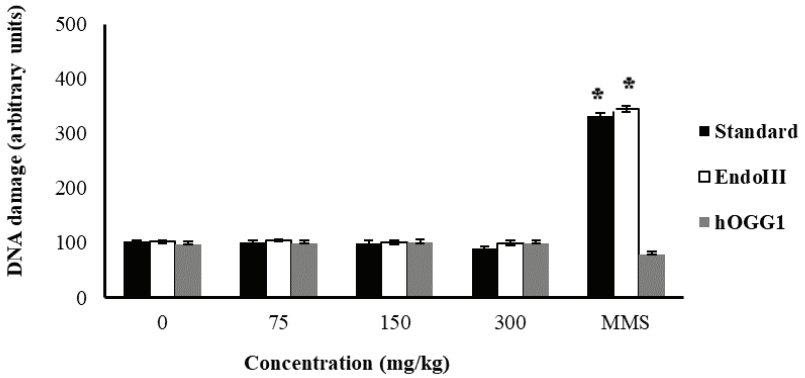

Figure 6 DNA damage induced by Fe_2O_3 nanoparticles in mice bone marrow measured by the standard and enzyme-modified comet assays. * significant difference ($P<0.05$) from negative control. EndoIII – endonuclease III-modified comet assay; hOGG1 – human 8-hydroxyguanine DNA-glycosylase-modified comet assay; MMS – methyl methanesulphonate. Note: the reason for low hOGG1 findings with MMS is that it cannot detect alkylating damage caused by it (43)

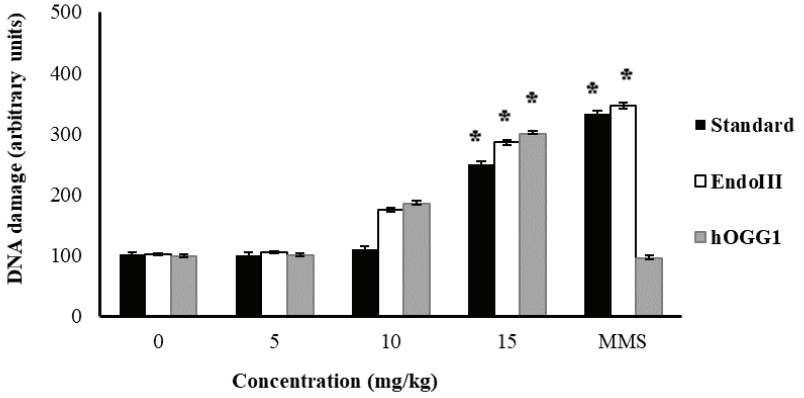

Figure 7 DNA damage induced by Cu nanoparticles in mice bone marrow measured by the standard and enzyme-modified comet assays. * significant difference ($P<0.05$) from negative control. EndoIII – endonuclease III-modified comet assay; hOGG1 – human 8-hydroxyguanine DNA-glycosylase-modified comet assay; 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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Genotoksičnost nanočestica aluminijeva oksida, željeznog oksida i bakra u mišjim stanicama koštane srži

Cilj ovog istraživanja bio je ocijeniti genotoksično djelovanje nanočestica Al_2O_3, Fe_2O_3 i Cu pomoću citogenetičkih testova kromosomskih aberacija, mikronukleus- i komet-testa u stanicama koštane srži muških BALB/c miševa. Miševi su intraperitonalnom injekcijom 14 dana primali jednu od triju doza Al_2O_3 ili Fe_2O_3 (75, 150 ili 300 mg/kg) odnosno Cu (5, 10 ili 15 mg/kg) nanočestica jednom na dan, dok je negativna kontrolna skupina primala destiliranu vodu a pozitivna mitomicin C i metil-metansulfonat. Nanočestice Al_2O_3 i Fe_2O_3 nisu iskazale genotoksično djelovanje, ali je zato bakar potaknuo značajnu ($P<0,05$) genotoksičnost u odnosu na negativnu kontrolu. Naši rezultati nadopunjuju spoznaje o rizicima za zdravlje povezanima s profesionalnom i drugom izloženosti (npr. putem potrošačke robe ili gdje se takve čestice primjenjuju u svrhu liječenja) nanočesticama Al_2O_3, Fe_2O_3 i Cu u ljudi te mogu poslužiti kao referenca pri utvrđivanju neškodljivih razina izloženosti u regulacijskim aktima. Potrebna su, međutim, daljnja istraživanja kronične toksičnosti ovih nanočestica *in vivo* kako bi se osigurala njihova sigurna primjena i spriječila štetna prisutnost u okolišu.

KLJUČNE RIJEČI: Al_2O_3; Cu; Fe_2O_3; *in vivo*; kromosomske aberacije; komet-test; mikronukleus; mitotski indeks