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In vivo genotoxicity assessment of aluminium oxide nanomaterials in rat peripheral blood cells using the comet assay and micronucleus test

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Advances in nanotechnology and its usage in various fields have led to the exposure of humans to engineered nanomaterials (NMs) and there is a need to tackle the potential human health effects before these materials are fully exploited. The main purpose of the current study was to assess whether aluminium oxide NMs (Al_2O_3-30 nm and Al_2O_3-40 nm) could cause potential genotoxic effects *in vivo*. Characterization of Al_2O_3-30 nm and Al_2O_3-40 nm was done with transmission electron microscopy, dynamic light scattering and laser Doppler velocimetry prior to their use in this study. The genotoxicity end points considered in this study were the frequency of micronuclei (MN) and the percentage of tail DNA (% Tail DNA) migration in rat peripheral blood cells using the micronucleus test (MNT) and the comet assay, respectively. Genotoxic effects were evaluated in groups of female Wistar rats (five per group) after single doses of 500, 1000 and 2000 mg/kg body weight (bw) of Al_2O_3-30 nm, Al_2O_3-40 nm and Al_2O_3-bulk. Al_2O_3-30 nm and Al_2O_3-40 nm showed a statistically significant dose-related increase in % Tail DNA for Al_2O_3-30 nm and Al_2O_3-40 nm ($P < 0.05$). However, Al_2O_3-bulk did not induce statistically significant changes over control values. The MNT also revealed a statistically significant ($P < 0.05$) dose-dependent increase in the frequency of MN, whereas Al_2O_3-bulk did not show any significant increase in frequency of MN compared to control. Cyclophosphamide (40 mg/kg bw) used as a positive control showed statistically significant ($P < 0.001$) increase in % Tail DNA and frequency of MN. The biodistribution of Al_2O_3-30 nm and Al_2O_3-40 nm and Al_2O_3-bulk in different rat tissues, urine and feces was also studied 14 days after treatment using inductively coupled plasma mass spectrometry. The data indicated that tissue distribution of Al_2O_3 was size dependent. Our findings suggest that Al_2O_3 NMs were able to cause size- and dose-dependent genotoxicity *in vivo* compared to Al_2O_3-bulk and control groups.

Introduction

Nanotechnology deals with the manipulation of materials at <100 nm and its exploitation in various fields, such as biology, medicine, pharmacology and electronics for the benefit of mankind. Metal and metal oxide nanomaterials (NMs) are used in cosmetics and skin care products, abrasives, polishers and as drug delivery systems. Nevertheless, the concerns over their potential human health impacts need to be addressed before they are fully accepted. NM toxicology is emerging as an important subdiscipline of nanotechnology, which could be the answer for the issues that are related to the possible toxic effects of NMs (1).

Currently, the most commercially important NMs are simple metal oxides, such as aluminium oxide (Al_2O_3), silica dioxide (SiO_2), titanium dioxide (TiO_2), iron oxide (Fe_3O_4, Fe_2O_3) and zinc oxide (ZnO). Among them, Al_2O_3 is the most abundantly produced NM, estimated to account for ~20% of the 2005 world market of NMs (2). The Al_2O_3 NMs have been widely used as abrasives, wear-resistant coatings on propeller shafts of ships, to increase the specific impulse per weight of composite propellants used in solid rocket fuel and as drug delivery systems to increase solubility (3–5). However, the risks posed by the Al_2O_3 NM due to their increased usage are little known and may be different from their bulk form, despite the chemical composition remaining the same. Implications of these NMs for cancer induction would promptly lead to risk and safety assessment and, as a result, genotoxicity studies are necessary.

A few studies have demonstrated that aluminium (Al) and Al compounds are genotoxic both *in vitro* and *in vivo* (6–8). Aluminium chloride evaluated with the micronucleus test (MNT), comet assay and chromosomal aberrations (CAs) analysis in human peripheral blood lymphocytes (PBLs) showed significant genotoxicity *in vitro* (9–11). *In vivo* studies with rats revealed that aluminium sulphate and potassium aluminium sulphate induced dose-dependent increases in CAs (12). There is a paucity of information on Al_2O_3 NM genotoxicity and it is a matter of concern whether these could cause genotoxic effects. In recent past, toxicity studies of NMs have mainly focused on cell culture systems and these could be misleading and need verification from animal experiments. The *in vivo* response of the NM possessing unique physico-chemical properties could lead to predictive models assessing toxicity (13). This prompted us to study the genotoxicity of Al_2O_3 NM (30 and 40nm) in rat peripheral blood (PB) cells using the comet assay and the MNT. Comet assay is a simple, inexpensive technique that evaluates chemicals for their ability to cause DNA strand breaks and alkali-labile sites under *in vivo* and *in vitro* conditions (14,15). The MNT provides a measure of both the clastogenicity and the aneugenicity of potential chemicals. It can be performed on rat PB cells and would be advantageous for evaluating genotoxic effects of chemicals as it allows multiple sampling in the rat without the necessity of sacrificing animals (16).

Toxicokinetic studies of NM can attribute to the cause of potential toxic effects of NM and their *in vivo* biodistribution in various organ systems and tissues is warranted. Systemic circulation could distribute the NM to all organs and tissues after the initial absorption of NM (17). Therefore, in the current

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investigation, the biodistribution of Al_2O_3-30 nm, Al_2O_3-40 nm and Al_2O_3-bulk in rat whole blood, liver, kidneys, heart, brain, spleen, urine and feces was analysed using inductively coupled plasma–mass spectrometry (ICP–MS). It is recommended that adequate NM characterization is needed prior to the initiation of toxicological experimentation to ascertain the possible cause for the toxicity of NM and in absence of this would have limited significance (18). Hence, in the present study the characterization of Al_2O_3-30 nm and Al_2O_3-40 nm was determined using transmission electron microscopy (TEM), dynamic light scattering (DLS) and laser Doppler velocimetry (LDV).

The purpose of this study was to analyze the possible dose-dependent genotoxic effects of Al_2O_3-30 nm, Al_2O_3-40 nm and Al_2O_3-bulk *in vivo* using the comet assay and the MNT. The study also tests the hypothesis of size-dependent genotoxicity of Al_2O_3-30 nm and Al_2O_3-40 nm compared to Al_2O_3-bulk.

Materials and methods

Chemicals

Al_2O_3-bulk [CAS number 1344-28-1, purchased from Sigma Aldrich Inc., USA; size: 70–290 mesh (50–200 μm); purity: >90%]. Al_2O_3-30 nm and Al_2O_3-40 nm (Product code: M1056, M1049-D, a gift from Dr Karl Martin of NovaCentrix, Austin, TX, USA; purity: >90%). Cyclophosphamide monohydrate (CPA) (CAS no. 6055-19-2) and acridine orange (AO) (CAS no. 10127-02-3) were obtained from Sigma Chemical Co. (St Louis, MO, USA). Phosphate-buffered saline (PBS) was purchased from Invitrogen, USA.

Characterization

TEM characterization was performed to obtain NM size and morphology on a Hitachi H-7600 tungsten-tip instrument at an accelerating voltage of 100 kV. NMs were examined after suspension in alcohol and subsequent deposition onto Formvar/carbon-coated TEM grids. The AMT software for the digital TEM camera was calibrated for size measurements of the NM. Information on mean size and standard deviation was calculated by measuring over 100 NMs in random fields of view in addition to images that show general morphology of the NM. TEM of Al_2O_3-30 nm (Figure 1) and Al_2O_3-40 nm (Figure 2) showed spherical morphologies and mean size distribution determined was 39.85 ± 31.33 nm and 47.33 ± 36.13 nm diameter, respectively.

Al_2O_3 NMs were suspended in 1% Tween 80, a surfactant in MilliQ water at stock concentration of 20% (w/v). The freshly prepared stock solutions were ultrasonicated using a bath sonicator for 10 min. DLS and LDV for size and charge characterization of Al_2O_3 NMs in solution were performed on a Malvern Instrument Zetasizer Nano ZS instrument. NMs were examined after suspension in 1% Tween 80 with and without ultrasonication at the stock concentration. However, the concentration was too high; hence, it was further diluted, adjusted to a lower concentration, for the device to acquire enough counts per second. Samples thus prepared were transferred to a 1.5-ml square cuvette for DLS measurements and 1 ml was transferred to a Malvern Clear Zeta Potential cell for LDV measurements. Average size was calculated by the software from the intensity, volume and number distributions measured. The polydispersity index given is a measure of the size ranges present in the solution with a scale ranging from 0 to 1, with 0 being monodisperse and 1 being polydisperse (19). The size and charge of Al_2O_3-30 nm and Al_2O_3-40 nm in solution using DLS and LDV, respectively, are presented in Table I.

Animals

Female Wistar rats (90–120 g) were used for the study. The animals were supplied by National Institute of Nutrition, Hyderabad, India. Rats were kept in polypropylene cages in an experimental room under controlled conditions of temperature (22 ± 3°C) and humidity (60 ± 10%), with feed and water being available *ad libitum*. Lighting was controlled to provide 12-h artificial light followed by 12 h darkness. The experiments were conducted in strict accordance with directive 86/609/EEC on the protection of laboratory animals. Institutional Animal Ethics Committee approved the study.

Experimental design

Freshly prepared suspensions of Al_2O_3-30 nm, Al_2O_3-40 nm and Al_2O_3-bulk were ultrasonicated for 10 min and mixed thoroughly prior to use. The rats were treated with 1 ml of each of the solutions for every 100 g of body weight (bw) and they received water and food *ad libitum* throughout the treatment period. To evaluate the genotoxicity of the Al_2O_3-30 nm, Al_2O_3-40 nm and Al_2O_3-bulk, the test substances were assessed at three different doses: 500, 1000 and 2000 mg/kg bw, via gavage. These doses were based on the acute oral toxicity study of Al_2O_3-30 nm, Al_2O_3-40 nm and Al_2O_3-bulk. The LD_{50} of these compounds was >2000 mg/kg bw (M. Mahboob, M.F. Rahman, N. Sailaja, A. Balasubramanyam, Sachin B. Agawane S. Hussain and Paramjit Grover unpublished data). The rats were divided into different groups of five for each treatment and these animals were the same for both the end points (comet assay and MNT). The first group, which served as a control group received the vehicle (1% Tween 80). The animals of the last group received a genotoxic agent CPA [40 mg/kg bw intraperitoneally (i.p.)]. Approximately 100 μl whole blood was collected from retro-orbital plexus at 4, 24, 48 and 72 h sampling times for the comet assay and at 48 and 72 h for MNT. To avoid bias, the slides were coded. One scorer analysed the slides throughout the study.

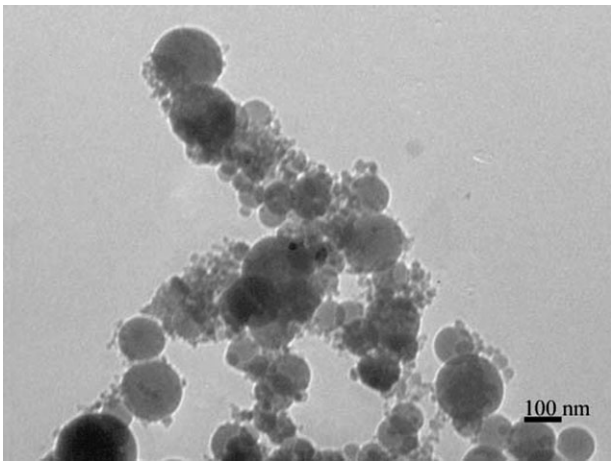

Fig. 1. TEM image for Al_2O_3-30 nm suspension in alcohol and dried on formvar/carbon-coated TEM grids.

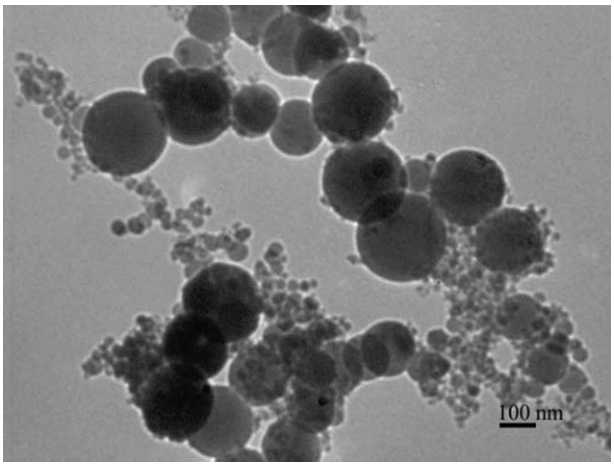

Fig. 2. TEM image for Al_2O_3-40 nm suspension in alcohol and dried on formvar/carbon-coated TEM grids.

Comet assay

The alkaline version of the comet assay was performed according to Singh *et al.* (14), with a slight modification. Cell viability was estimated by trypan blue exclusion assay (20). Slides were prepared in triplicate per sample per experiment. At 4, 24, 48 and 72 h time intervals after the treatment, 20 μl of heparinized PB was mixed with 110 μl of 0.5% low-melting–temperature agarose in PBS and applied to microscope slides pre-coated with 0.75% normal-melting–temperature agarose in PBS. The slides were covered with a microscope coverslip and refrigerated for 5 min to gel. This was followed by immersion in ice-cold alkaline lysing solution (2.5 M NaCl, 10 mM Tris, 100 mM EDTA, 10% dimethyl sulphoxide, 1% Triton X-100, final pH 10.0) for at least 1 h. The slides were then incubated for 20 min in ice-cold electrophoresis solution (0.3 M NaOH, 1 mM EDTA, pH > 13), followed by electrophoresis at 25 V: 300 mA (1.25 V/cm) for 25 min. After electrophoresis, the slides were

then neutralized (Tris 0.4 M, pH 7.5) and stained with ethidium bromide (20 μg/ml). One hundred and fifty cells per rat (50 cells analysed on each slide) were scored at 400× using a fluorescence microscope (Olympus-Japan) with a blue (488 nm) excitation filter and yellow (515 nm) emission (barrier) filter. Quantification of DNA breakage was realized using a Comet Image Analysis System, version 5.5 (Kinetic Imaging Ltd, Nottingham, UK). Results were reported as % Tail DNA (21).

MNT

The MNT was performed on PB cells according to the protocol described by Celik *et al*. (16) and according to the test guideline Organization for Economic Co-operation and Development (OECD) 474 (22). Whole blood was collected at 48 and 72 h after dose and smears were prepared on clean microscope slides, air-dried, fixed in methanol and stained with AO (125 μg/ml in pH 6.8 phosphate buffer) for 1 min just before the evaluation with a fluorescence microscope using a 100× objective. The frequency of polychromatic erythrocytes (PCEs) per total erythrocytes was determined using a sample size of 1000 erythrocytes per animal. The number of micronucleated PCEs was determined using 2000 PCEs per animal.

Aluminium oxide content analysis

The animals were sacrificed 14 days after a single oral dose of 500, 1000 or 2000 mg/kg bw of Al_2O_3-30 nm, Al_2O_3-40 nm or Al_2O_3-bulk for collection of whole blood, liver, kidneys, heart, brain and spleen. Urine and feces samples were collected 48 h after the dosing. About 0.1–0.3 g of fresh whole blood, liver, kidneys, heart, brain, spleen and feces samples from the treated rats were predigested in nitric acid (ultrapure grade) overnight. Samples were then heated at 80°C for 10 h followed by additional heating at 130–150°C for 30 min. Finally, in the presence of 0.5 ml of 70% perchloric acid, the samples were again heated for 4 h, and evaporated nearly to dryness (23). Subsequently, these solutions and the urine (0.5 ml) samples were made up to 5 ml with deionized water and filtered. ICP–MS (ELAN DRC II, Perkin Elmer Sclex.) was used to analyse the Al_2O_3 concentration in the samples. Rhodium at 20 ng/ml was used as an internal standard element.

Data evaluation

Data are presented as means and standard deviations and the means of individual animals or experimental units were compared by one-way analysis of variance and multiple pair-wise comparisons were done using the Tukey's test. Group mean values of each experiment were also compared by single-sided Student's *t*-test. $P < 0.05$ was considered to be the level of significance. Statistical analysis was performed using Graph Pad Prism 3 Software package for Windows.

Results

In the current investigation, size of Al_2O_3 NM was measured with TEM and was found to match manufacturer's data (30 and 40 nm). However, the DLS data revealed the aggregation of Al_2O_3-30 nm and Al_2O_3-40 nm in suspensions, which could be possibly due to physico-chemical interactions between the NMs. Hence, in order to create a homogenous solution, constant re-suspension is necessary prior to use. Previous studies on characterization of Al_2O_3 NMs showed similar results, the aggregation of the NM in water and different cell culture media with or without serum (19,24).

Cell viability assessed in blood cells after treatment with all three doses (500, 1000 and 2000 mg/kg bw) of each of the Al_2O_3-30 nm, Al_2O_3-40 nm and Al_2O_3-bulk showed high percentage of viability (>80%) at all sampling times. Comet assay results showed statistically significant increase in % Tail DNA (Table II) after treatment with Al_2O_3-30 nm and Al_2O_3-40 nm in comparison to the control group. In the present

Table I. Al_2O_3 NM size and charge in solution

Nanomaterials	DLS		LDV		
	Average diameter (nm)	PDI	Zeta potential ζ (mV)	Electrophoretic mobility μ (μm·cm/V/s)	pH
Al_2O_3-30 nm					
MilliQ water	212.0	0.132	25.2	2.221	7.0
1% Tween 80	184.7	0.199	15.3	1.410	7.0
1% Tween 80 sonicated	138.8	0.280	50.5	4.006	6.5
Al_2O_3-40 nm					
MilliQ water	226.1	0.152	20.3	2.410	7.0
1% Tween 80	194.6	0.343	9.8	0.774	7.0
1% Tween 80 sonicated	147.3	0.390	41.6	3.216	6.5

Nanomaterials were dispersed in MilliQ water, 1% Tween 80. Mixing was done via bath sonication and vortexing.

Table II. Percent comet tail DNA (mean ± SD) calculated from 150 cells per rat ($n = 5$ rats per group) exposed to different doses of Al_2O_3 NM and bulk groups at various time intervals

Treatments	Dose (mg/kg bw)	Sampling times (h)			
		4	24	48	72
Tween 80	1% (v/v)	3.38 ± 1.94	5.88 ± 3.07	4.02 ± 2.84	2.22 ± 1.08
Al_2O_3-30 nm	500	13.06 ± 6.03	11.92 ± 3.99	7.50 ± 3.78	3.60 ± 1.92
	1000	27.06 ± 8.41**	24.78 ± 10.15**	15.18 ± 4.80*	5.70 ± 3.70
	2000	34.78 ± 14.00**	42.78 ± 13.98***	23.78 ± 7.92**	8.78 ± 3.73
Al_2O_3-40 nm	500	10.62 ± 6.89	8.31 ± 5.32	5.55 ± 2.49	2.43 ± 1.82
	1000	20.00 ± 8.08*	20.78 ± 9.23*	10.58 ± 6.51	3.96 ± 2.46
	2000	26.80 ± 9.20*	34.38 ± 12.68**	18.38 ± 9.53*	6.58 ± 2.80
Al_2O_3-bulk	500	2.42 ± 1.14	4.68 ± 2.44	3.12 ± 1.16	2.09 ± 0.99
	1000	3.80 ± 2.61	5.26 ± 2.48	2.80 ± 1.37	2.12 ± 1.18
	2000	5.40 ± 3.59	7.26 ± 4.53	4.46 ± 1.71	2.66 ± 1.44
CPA	40	52.73 ± 13.75***	59.73 ± 16.76***	31.73 ± 10.59***	19.13 ± 5.95*

SD: standard deviation; CPA: positive control; 1% Tween 80: solvent control.
*$P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

experiment, statistically significant differences in % Tail DNA were seen in groups of animals sampled at all time intervals after treatment with 2000 mg/kg bw Al_2O_3-30 nm and the animals sampled 4, 24 and 48 h after treatment with the same dose of Al_2O_3-40 nm. The groups of animals sampled 4, 24 and 48 h after treatment with 1000 mg/kg bw Al_2O_3-30 nm and the animals sampled 4 and 24 h after treatment with the same dose level Al_2O_3-40 nm, showed statistically significant enhancement in % Tail DNA. No statistically significant increase in % Tail DNA was observed after treatment with the lowest dose of 500 mg/kg bw Al_2O_3-30 nm and Al_2O_3-40 nm sampled at all time intervals. In contrast, the groups of animals sampled at all time intervals after treatment with three doses (500, 1000 and 2000 mg/kg bw) Al_2O_3-bulk did not show statistically significant increases in % Tail DNA compared to the control group. A clear induction of DNA damage was observed in the animals sampled at all time intervals after treatment with 40 mg/kg bw CPA.

MNT data indicated statistically significant effects on micronuclei (MN) frequency (Table III) after treatment with Al_2O_3-30 nm and Al_2O_3-40 nm compared to the control group. Groups of animals sampled 48 and 72 h after treatment with 1000 and 2000 mg/kg bw Al_2O_3-30 nm and Al_2O_3-40 nm revealed a significant increase in MN frequency but not with 500 mg/kg bw. However, treatment with all three doses (500, 1000 and 2000 mg/kg bw) of Al_2O_3-bulk did not evoke a significant increase in MN frequency in the groups of animals sampled at both time intervals. The percentage PCEs calculated after treatment with all three doses of Al_2O_3-30 nm, Al_2O_3-40 nm and Al_2O_3-bulk did not show statistically significant differences compared to the control at both the sampling times. CPA (40 mg/kg bw) induced a substantially significant effect on MN frequency.

Al_2O_3 accumulated in all tissues viz., whole blood, liver, heart, kidneys, brain and spleen in the groups of animals sampled 14 days after treatment with all three doses (500, 1000 and 2000 mg/kg bw) of Al_2O_3-30 nm, Al_2O_3-40 nm and Al_2O_3-bulk (Table IV). The biodistribution of Al_2O_3 showed statistically significant increases in different tissues and urine after treatment with 1000 and 2000 mg/kg bw Al_2O_3-30 nm and Al_2O_3-40 nm compared to the control. The maximum amount of accumulation was seen in the kidneys followed by whole blood, liver and brain of animals after treatment with all three doses of Al_2O_3-30 nm and Al_2O_3-40 nm. However, it is

Table III. Frequency of micronucleated polychromatic erythrocytes for a total of 2000 analyzed cells per rat ($n = 5$ rats per group) in each treatment to evaluate the genotoxicity of different doses of Al_2O_3 NM and bulk groups

Treatments	Dose (mg/kg bw)	48 h		72 h	
		MNPCEs (mean ± SD)	%PCEs	MNPCEs (mean ± SD)	%PCEs
Tween 80	1% (v/v)	1.51 ± 0.93	3.43	1.63 ± 0.83	4.12
Al_2O_3-30 nm	500	2.77 ± 1.23	3.21	2.89 ± 1.45	3.67
	1000	8.20 ± 2.17*	2.92	6.12 ± 1.86*	3.32
	2000	15.81 ± 3.45**	3.02	9.21 ± 2.10**	2.99
Al_2O_3-40 nm	500	2.45 ± 1.56	2.88	2.67 ± 1.78	3.06
	1000	7.51 ± 2.25*	2.45	5.45 ± 1.65*	3.79
	2000	12.08 ± 3.18**	2.98	8.31 ± 2.47**	2.85
Al_2O_3-bulk	500	1.75 ± 0.67	3.18	1.56 ± 0.76	3.54
	1000	1.98 ± 0.98	2.96	2.10 ± 1.12	3.02
	2000	3.99 ± 1.29	2.30	2.40 ± 1.39	2.77
CPA	40	28.2 ± 7.10***	1.52*	25.41 ± 8.20***	2.38

SD: standard deviation; CPA: positive control; 1% Tween 80: solvent control.
*$P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

Table IV. Al_2O_3 content in different tissues, urine and feces of rats treated with Al_2O_3 NM and bulk groups

Samples analyzed for Al_2O_3 content	Concentration of Al_2O_3 in tissues, urine and feces of treatment groups									
	Control	Al_2O_3-30 nm dose (mg/kg bw)			Al_2O_3-40 nm dose (mg/kg bw)			Al_2O_3-bulk dose (mg/kg bw)		
		500	1000	2000	500	1000	2000	500	1000	2000
Whole blood[a]	3 ± 1	47 ± 16	79 ± 25**	156 ± 21***	45 ± 14	73 ± 26*	129 ± 17***	12 ± 4	32 ± 9	76 ± 9
Liver[a]	6 ± 2	56 ± 18	84 ± 12*	158 ± 23***	42 ± 11	78 ± 18**	119 ± 21***	22 ± 18	52 ± 15	71 ± 11
Spleen[a]	2 ± 1	28 ± 21	59 ± 22*	153 ± 17***	37 ± 16	60 ± 13**	116 ± 11***	15 ± 7	28 ± 7	51 ± 10
Heart[a]	6 ± 3	37 ± 11	69 ± 29*	121 ± 13***	35 ± 21	66 ± 14*	102 ± 18***	14 ± 6	25 ± 9	57 ± 10
Kidneys[a]	9 ± 4	62 ± 19*	122 ± 20**	231 ± 29***	48 ± 15*	92 ± 18**	156 ± 22***	21 ± 7	34 ± 16	63 ± 20
Brain[a]	2 ± 1	50 ± 21	109 ± 22**	187 ± 19***	35 ± 17	90 ± 23*	151 ± 13***	14 ± 3	23 ± 6	59 ± 11
Urine[b]	5 ± 2	48 ± 15*	95 ± 17**	191 ± 16***	44 ± 9	80 ± 17**	183 ± 18***	9 ± 5	19 ± 8	54 ± 12
Faeces[c]	1 ± 1	4 ± 3	7 ± 3	21 ± 5	5 ± 1	8 ± 2	27 ± 6	16 ± 7	26 ± 7	37 ± 9**

Data represented as mean ± SD.
[a]Values represented as μg/g tissues.
[b]Values represented as μg/ml urine.
[c]Values represented as mg/g faeces.
*$P < 0.05$, **$P < 0.01$, ***$P < 0.001$.

evident from Table IV that Al_2O_3-bulk did not show statistically significant accumulation in any of the tissues and urine compared to the control. Nevertheless, it showed largest retention in feces.

Discussion

The results obtained in the comet assay indicated that Al_2O_3-30 nm and Al_2O_3-40 nm were able to cause a significant and dose-dependent increase in % Tail DNA migration compared to Al_2O_3-bulk and control groups at all time intervals. However, Al_2O_3-30 nm and Al_2O_3-40 nm groups showed a gradual time-dependent decrease of % Tail DNA. This decline could be due to modification of the DNA lesions by DNA repair processes in a complex manner (25). Hence, the decrease in the DNA damage can be interpreted as a consequence of DNA repair. Our study revealed significant dose-dependent increases in the MN frequency in PB indicating possible chromosomal changes with the Al_2O_3-30 nm and Al_2O_3-40 nm at 48 and 72 h sampling times in comparison with the Al_2O_3-bulk and control groups. Nevertheless, the MN frequency induced by the Al_2O_3-30 nm and Al_2O_3-40 nm was reduced with increase in exposure time. These results were consistent with the comet assay data, which also showed time-dependent decrease in DNA damage. Further, it was revealed that the percent PCEs calculated in Al_2O_3-30 nm, Al_2O_3-40 nm and Al_2O_3-bulk treated groups did not show any significant decrease compared to the control group suggesting that cell death had not occurred in any of the treated groups.

The results from this study indicated that Al_2O_3-30 nm was most genotoxic and the bulk effects were insignificant. Likewise, Colognato *et al*. (26) reported that cobalt (Co) NM can significantly increase the frequency of binucleated MN cells and % Tail DNA studied in human PBL whereas Co^{2+} ions were not genotoxic *in vitro*. It was also found that ultrafine TiO_2 induced MN significantly in Syrian hamster embryo cells. In contrast, fine TiO_2 did not induce MN to a significant extent (27). The genotoxicity observed with Al_2O_3 NM may be due to pro-inflammatory effects through a reactive oxygen species (ROS)-mediated mechanism (28). Based on the present findings, we support the hypothesis that the size of the Al_2O_3 NM may be the cause of the significant genotoxicity.

Our data are in agreement with an *in vitro* genotoxicity study of TiO_2, Al_2O_3 and gold NM, investigated in A549, HEPG2 and NRK-52E cells with the MNT, comet and gamma-H2AX histone foci quantification (29). Our study is also in accordance with the investigations of TiO_2 NM using cultured human MRC-5 fibroblasts and WIL2-NS lymphoblastoid cells, which showed pronounced genotoxicity in the cytokinesis block MNT, the comet assay and the hypoxanthine–guanine phosphoribosyl transferase gene mutation assay (30,31). Similarly, genotoxicity investigated with magnetite NM using the MNT in mice revealed a genotoxic effect (32). In contrast, *in vivo* genotoxicity studied in rats exposed to silver NM for 28 days showed no significant increase of MNPCEs in bone marrow (33). Furthermore, the results obtained with NM of TiO_2-P25 and T805 did not show significant differences compared to control in the amount of 8-oxoGua as a marker of DNA damage in NM-instilled rat lung tissue (34). Likewise, magnetic NM evaluated for genotoxicity increased the number of revertants in the Ames assay, but the mutation pattern was neither reproducible nor concentration dependent. Moreover, these NM did not induce any significant CA in Chinese hamster lung fibroblast cells (35).

In the current study the size- and dose-dependent distribution of Al_2O_3-30 nm, Al_2O_3-40 nm and Al_2O_3-bulk was detected in various tissues, urine and feces with the ICP–MS measurements. The absorption of these NMs could be across the gastrointestinal tract and passage through the lymph and lymph nodes to different tissues. Al_2O_3-30 nm and Al_2O_3-40 nm showed maximum quantity in kidneys, whole blood, liver and brain, whereas Al_2O_3-bulk distribution was significantly lower than Al_2O_3-30 nm and Al_2O_3-40 nm in all tissues but it showed higher amounts in the feces than Al_2O_3-30 nm and Al_2O_3-40 nm, indicating that size is a barrier to absorption of Al_2O_3 in rats. Our results also indicated that there was maximum accumulation of Al_2O_3-30 nm. Significant retention of Al_2O_3-30 nm and Al_2O_3-40 nm in the whole blood compared to control could be imputed to the possible genotoxic effects. Besides, Al_2O_3 NM showed increased amount of retention in kidneys, which could be attributed to the entrapment of these NMs in the reticular endothelial system and excreted by the kidneys *in vivo*. Our ICP–MS data also showed the accumulation of Al_2O_3 NM in brain tissue but did not confirm or refute the possibility of blood brain barrier (BBB) passage.

Our results are supported by the fact that 0.1% of ingested Al is usually absorbed following oral exposure, although absorption of more bioavailable forms can be on the order of 1%. The unabsorbed Al is excreted in the feces. The mechanism of absorption is probably passive diffusion through paracellular pathways. Al binds to various ligands in the blood and distributes to every organ, with highest concentrations found in bone and lung tissues. Absorbed Al is excreted principally in the urine and, to a lesser extent, in the bile (36).

The sampling times for biodistribution and genotoxicity studies were different. The samples for biodistribution study were collected after 14 days of oral exposure. Similarly, biodistribution of different-sized TiO_2 particles was conducted 2 weeks after oral administration (37). The experimental design of the genotoxicity study was in accordance with guidelines for the comet assay (15) and the MNT (22).Our results are in agreement with the size-dependent distribution of gold NM to whole blood, liver, brain, spleen, kidneys and lungs after intravenous administration (17). Similarly, micro-copper did not accumulate in any of the organs of mice following a single oral gavage treatment, whereas copper NM showed accumulation in liver, kidneys and blood (38). Moreover, a study on magnetic NM revealed tissue distribution in various organs including brain of mice showed that circumventricular organs within the brain are not protected by the BBB (35). Similar kinetic studies in mice with different nano-sized TiO_2 particles showed its retention in the liver, spleen, kidneys and lung tissues (37).

The Al_2O_3-bulk content in whole blood at a dose level of 2000 mg/kg bw was similar to the amounts of Al_2O_3 NM at 1000 mg/kg bw. Nevertheless, Al_2O_3-bulk did not show significant genotoxic effects compared to control. Toxic effects of NMs are specific to the type of base material, size, ligands and coatings. It has been observed that NMs show greater toxicity than fine particulates of the same material on a mass basis. This has been seen with the different types of NMs, including TiO_2, Al_2O_3, carbon black, Co and nickel (39). Hence, it may be concluded that the significant genetic damage of Al_2O_3 NM could be due to their nanoscale size. However, further studies are imperative before any staunch conclusions could be drawn regarding the comparison of possible genotoxic effects of Al_2O_3 NM and Al_2O_3-bulk. The genotoxicity of Al seems to be via three mechanisms: modification of chromatin

structure, induction of ROS and liberation of DNase from the lysosomes (9). The first mechanism is corroborated by the findings that Al can influence the structure of chromatin and would lead to DNA damage (40,41). The second mechanism is substantiated by the observations that interaction of cells with Al can lead to the formation of ROS (42,43). A few studies confirmed the last mechanism that Al can enhance the permeability of the lysosomal membrane and inhibit the lysosomal proton pump, which could lead to DNase being liberated into the cytoplasm and its passage into the nucleus, where it would cut DNA (44,45). It has been shown that DNase introduced into the cytoplasm by electroporation is a potent inducer of cytogenetic damage (46). It is conceivable that Al induces genotoxicity by one or all of these mechanisms. Further studies are necessary before any firm conclusion can be drawn.

All together, our results suggest that Al_2O_3 NMs are able to cause genotoxic effects *in vivo* and, to our knowledge, this is the first genotoxicity study of Al_2O_3 NM in rats. Nevertheless, there is a need to better understand the molecular mechanisms involved in the genotoxicity of Al_2O_3 NM for its safer and proper utilization.

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References

1. Oberdorster, G., Oberdorster, E. and Oberdorster, J. (2005) Nanotoxicology: an emerging discipline evolving from studies of ultrafine particles. *Environ. Health Perspect.*, **113**, 823–839.
2. Rittner, M. N. (2002) Market analysis of nanostructured materials. *Am. Ceram. Soc. Bull.*, **81**, 33–36.
3. Miziolek, A. (2002) Nanoenergetics: an emerging technology area of national importance. *AMPTIAC Q.*, **6**, 43–48.
4. Department of Defense Director. (2005) Defense Research and Engineering. (2005) Defense Nanotechnology Research and Development. (2006). http://www.nano.gov/html/res/DefenseNano2005.pdf (last accessed date: 4 January 2006).
5. Tyner, K. M., Schiffman, S. R. and Giannelis, E. P. (2004) Nanobiohybrids as delivery vehicles for camptothecin. *J. Control Release*, **95**, 501–514.
6. Manna, G. K. and Das, R. K. (1972) Chromosome aberrations in mice induced by aluminium chloride. *Nucleus*, **15**, 180–186.
7. Wedrychowski, A., Schmidt, W. N. and Hnilica, L. S. (1986) The *in-vivo* cross-linking of proteins and DNA by heavy metals. *J. Biol. Chem.*, **261**, 3370–3376.
8. Blair, H. C., Finch, J. L., Avioli, R., Crouch, E. C., Slatopolsky, E. and Teitelbaum, S. L. (1989) Micromolar aluminium levels reduce 3H-thymidine incorporation by cell line UMR 106-01. *Kidney Int.*, **35**, 1119–1125.
9. Banasik, A., Lankoff, A., Piskulak, A., Adamowska, K., Lisowska, H. and Wojcik, A. (2005) Aluminium-induced micronuclei and apoptosis in human peripheral blood lymphocytes treated during different phases of the cell cycle. *Environ. Toxicol.*, **20**, 402–406.
10. Lankoff, A., Banasik, A., Duma, A., Ochniak, E., Lisowska, H., Kuszewski, T., Góźdź, S. and Wojcik, A. (2006) A comet assay study reveals that aluminium induces DNA damage and inhibits the repair of radiation-induced lesions in human peripheral blood lymphocytes. *Toxicol. Lett.*, **161**, 27–36.
11. Lima, P. D. L., Leite, D. S., Vasconcellos, M. C., Cavalcanti, B. C., Santos, R. A., Costa-Lotufo, L. V., Pessoa, C., Moraes, M. O. and Burbano, R. R. (2007) Genotoxic effects of aluminium chloride in cultured human lymphocytes treated in different phases of cell cycle. *Food Chem. Toxicol.*, **45**, 1154–1159.
12. Roy, A. K., Sharma, A. and Talukder, G. (1991) Effects of aluminium salts on bone marrow chromosomes in rats *in vivo*. *Cytobios*, **66**, 105–111.
13. Fischer, H. C. and Chan, W. C. W. (2007) Nanotoxicity: the growing need for *in vivo* study. *Curr. Opin. Biotechnol.*, **8**, 565–571.
14. Singh, N. P., McCoy, M. T., Tice, R. R. and Schneider, E. L. (1988) A simple technique for quantitation of low levels of DNA damage in individual cells. *Exp. Cell Res.*, **175**, 184–191.
15. Tice, R. R., Agurell, E., Anderson, D. *et al.* (2000) The single cell gel/comet assay: guidelines for *in vitro* and *in vivo* genetic toxicology testing. *Environ. Mol. Mutagen.*, **35**, 206–221.
16. Celik, A., Ogenler, O. and Cömelekoglu, U. (2005) The evaluation of micronucleus frequency by acridine orange fluorescent staining in peripheral blood of rats treated with lead acetate. *Mutagenesis*, **20**, 411–415.
17. De Jong, W. H., Hagens, W. I., Krystek, P., Burger, M. C., Sips, A. J. A. M. and Geertsma, R. E. (2008) Particle size-dependent organ distribution of gold nanoparticles after intravenous administration. *Biomaterials*, **29**, 1912–1919.
18. Warheit, D. B. (2008) How meaningful are the results of nanotoxicity studies in the absence of adequate material characterization? *Toxicol. Sci.*, **101**, 183–185.
19. Wagner, A. J., Bleckmann, C. A., Murdock, R. C., Schrand, A. M., Schlager, J. J. and Hussain, S. M. (2007) Cellular interaction of different forms of aluminium nanoparticles in rat alveolar macrophages. *J. Phys. Chem. Biol.*, **111**, 7353–7359.
20. Pool-Zobel, B. L., Lotzmann, N., Knoll, M., Kuchenmeister, F., Lambertz, R., Leucht, U., Schroder, H. G. and Schmezer, P. (1994) Detection of genotoxic effects in human gastric and nasal mucosa cells isolated from biopsy samples. *Environ. Mol. Mutagen.*, **24**, 23–45.
21. Lovell, D. P. and Omori, T. (2008) Statistical issues in the use of the comet assay. *Mutagenesis*, **23**, 171–182.
22. OECD (1997) *Guideline for the Testing of Chemicals: Mammalian Erythrocyte Micronucleus Test. Guideline 474*. Organisation for Economic Cooperation and Development, Paris, France.
23. Gómez, M., Sánchez, D. J., Llobet, J. M., Corbella, J. and Domingo, J. L. (1997) The effect of age on aluminium retention in rats. *Toxicology*, **116**, 1–8.
24. Murdock, R. C., Braydich-Stolle, L., Schrand, A. M., Schlager, J. J. and Hussain, S. M. (2008) Characterization of nanomaterial dispersion in solution prior to *in vitro* exposure using dynamic light scattering technique. *Toxicol. Sci.*, **101**, 239–253.
25. Hartmann, A. and Speit, G. (1995) Genotoxic effects of chemicals in the single cell gel (SCG) test with human blood cells in relation to the induction of sister-chromatid exchanges (SCE). *Mutat. Res.*, **346**, 49–56.
26. Colognato, R., Bonelli, A., Ponti, J., Farina, M., Bergamaschi, E., Sabbioni, E. and Migliore, L. (2008) Comparative genotoxicity of cobalt nanoparticles and ions on human peripheral leukocytes *in vitro*. *Mutagenesis*, **23**, 377–382.
27. Rahman, Q., Lohani, M., Dopp, E., Pemsel, H., Jonas, L., Weiss, D. G. and Schiffmann, D. (2002) Evidence that ultrafine titanium dioxide induces micronuclei and apoptosis in Syrian hamster embryo fibroblasts. *Environ. Health Perspect.*, **110**, 797–800.
28. Oesterling, E., Chopra, N., Gavalas, V., Arzuaga, X., Lim, E. J., Sultana, R., Butterfield, D. A., Bachas, L. and Hennig, B. (2008) Alumina nanoparticles induce expression of endothelial cell adhesion molecules. *Toxicol. Lett.*, **178**, 160–166.
29. Simon, A., Gouget, B., Mayne, M., Herlin, N., Reynaud, C., Degrouard, J. and Carriere, M. (2007) *In vitro* investigation of TiO_2, Al_2O_3, Au nanoparticles and multi-walled carbon nanotubes cyto- and genotoxicity on lung, kidney cells and hepatocytes. *Toxicol. Lett.*, **172**, S36.
30. Dunford, R., Salinaro, A., Cai, L., Serpone, N., Horikoshi, S., Hidaka, H. and Knowland, J. (1997) Chemical oxidation and DNA damage catalysed by inorganic sunscreen ingredients. *FEBS Lett.*, **418**, 87–90.
31. Wang, J. J., Sanderson, B. J. S. and Wang, H. (2007) Cyto- and genotoxicity of ultrafine TiO_2 particles in cultured human lymphoblastoid cells. *Mutat. Res.*, **628**, 99–106.
32. Sadeghiani, N., Barbosa, L. S., Silva, L. P., Azevedo, R. B., Morais, P. C. and Lacavaa, Z. G. M. (2005) Genotoxicity and inflammatory investigation in mice treated with magnetite nanoparticles surface coated with polyaspartic acid. *J. Magn. Magn. Mater.*, **289**, 466–468.
33. Kim, Y. S., Kim, J. S., Cho, H. S. *et al.* (2008) Twenty-eight-day oral toxicity, genotoxicity, and gender-related tissue distribution of silver nanoparticles in Sprague-Dawley rats. *Inhal. Toxicol.*, **20**, 575–583.

34. Rehn, B., Seiler, F., Rehn, S., Bruch, J. and Maier, M. (2003) Investigations on the inflammatory and genotoxic lung effects of two types of titanium dioxide: untreated and surface treated. *Toxicol. Appl. Pharmacol.*, **189**, 84–95.
35. Kim, J. S., Yoon, T. J., Yu, K. N. *et al.* (2006) Toxicity and tissue distribution of magnetic nanoparticles in mice. *Toxicol. Sci.*, **89**, 338–347.
36. ATSDR (2006) *Toxicological Profile of Aluminium*. Agency for Toxic Substances and Disease Registry (ATSDR), Department of Health and Human Services (HHS).
37. Wang, J., Zhou, G., Chen, C. *et al.* (2007) Acute toxicity and biodistribution of different sized titanium dioxide particles in mice after oral administration. *Toxicol. Lett.*, **168**, 176–185.
38. Chen, Z., Meng, H., Yuan, H., Xing, G., Chen, C., Zhao, F., Wang, Y., Zhang, C. and Zhao, Y. (2007) Identification of target organs of copper nanoparticles with ICP-MS technique. *J. Radioanal. Nucl. Chem.*, **272**, 599–603.
39. Hallock, M. F., Greenley, P., DiBerardinis, L. and Kallin, D. (2009) Potential risks of nanomaterials and how to safely handle materials of uncertain toxicity. *J. Chem. Health Safety*, **16**, 16–23.
40. Hanas, J. S. and Gunn, C. G. (1996) Inhibition of transcription factor IIIA–DNA interactions by xenobiotic metal ions. *Nucl. Acids Res.*, **24**, 924–930.
41. Bharathi Jagannatha Rao, K. S. and Stein, R. (2003) First evidence on induced topological changes in supercoiled DNA by an aluminium D-aspartate complex. *J. Biol. Inorg. Chem.*, **8**, 823–830.
42. Anane, R. and Creppy, E. E. (2001) Lipid peroxidation as pathway of aluminium cytotoxicity in human skin fibroblast cultures: prevention by superoxide dismutase+catalase and vitamins E and C. *Hum. Exp. Toxicol.*, **20**, 477–481.
43. Moumen, R., Ait-Oukhatar, N., Fleury, C., Bougle, D., Arhan, P., Neuville, D. and Viader, F. (2001) Aluminium increases xanthine oxidase activity and disturbs antioxidant status in the rat. *J. Trace Elements Med. Biol.*, **15**, 89–93.
44. van der Voet, G. B., Brandsma, A. E., Heijink, E. and de Wolff, F. A. (1992) Accumulation of aluminium in rat liver: association with constituents of the cytosol. *Pharmacol. Toxicol.*, **70**, 173–176.
45. Zatta, P., Taylor, A., Zambenedetti, P., Milacic, R. and dell'Antone, P. (2000) Aluminium inhibits the lysosomal proton pump from rat liver. *Life Sci.*, **66**, 2261–2266.
46. Sayed Aly, M., Wojcik, A., Schunck, C. and Obe, G. (2002) Correlation of chromosomal aberrations and sister chromatid exchanges in individual CHO cells pre-labelled with BrdU and treated with DNaseI or X-rays. *Int. J. Radiat. Biol.*, **78**, 1037–1044.

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