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RESEARCH ARTICLE

Genotoxic effects of chromium oxide nanoparticles and microparticles in Wistar rats after 28 days of repeated oral exposure

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Abstract The nanotechnology industry has advanced rapidly in the last 10 years giving rise to the growth of the nanoparticles (NPs) with great potential in various arenas. However, the same properties that make NPs interesting raise concerns because their toxicity has not been explored. The in vivo toxicology of chromium oxide (Cr_2O_3)–NPs is not known till date. Therefore, this study investigated the 28-day repeated toxicity after 30, 300 and 1000 mg/kg body weight (bw)/day oral treatment with Cr_2O_3–NPs and Cr_2O_3 microparticles (MPs) in Wistar rats. The mean size of Cr_2O_3–NPs and Cr_2O_3–MPs was 34.89±2.65 nm and 3.76±3.41 μm, respectively. Genotoxicity was assessed using comet, micronucleus and chromosomal aberration (CA) assays. The results revealed a significant increase in DNA damage in peripheral blood leucocytes and liver, micronuclei and CA in bone marrow after exposure of 300 and 1000 mg/kg doses of Cr_2O_3–NPs and Cr_2O_3–MPs only at 1000 mg/kg bw/day. Cr biodistribution was observed in all the tissues in a dose-dependent manner. The maximum amount of Cr was found in the kidneys and least in the brain of the treated rats. More of the Cr was excreted in the faeces than in the urine. Furthermore, nanotreated rats displayed much higher absorption and tissue accumulation. These findings provide initial data of the probable genotoxicity and biodistribution of NPs and MPs of Cr_2O_3 generated through repeated oral treatment.

Keywords Chromium oxide nanoparticles · Characterization · Comet · Micronucleus test · Chromosomal aberrations · Biodistribution

Introduction

Nanotechnology has advanced to a sizable extent during the last 20 years. Currently, nanoparticles (NPs) are being used in various consumer products ranging from aerospace engineering to everyday household goods and in medical sciences for diagnostics, imaging and drug delivery (Kim and Hyeon 2014). Because of their small size, NPs have a large surface area and consequently more surface atoms than large bulk particles. Hence, NPs are capable of entering the human body by inhalation, ingestion, skin penetration or injection routes and interact with intracellular structures for long period of time (Nel et al. 2006). Since the methodologies for exposure assessment are not consistent, research on toxicology of engineered NPs is largely lacking. Consequently, the human health and environmental safety of NPs has drawn increasing public and government attention and the scarcity of the information on the toxicology of NPs is a major concern. Therefore, in order to reduce the considerable knowledge gap between development and toxicity of NPs, a major effort is needed by the scientific community to study the effects of exposure to NPs.

In recent years, great interest has been shown in chromium oxide (Cr_2O_3)–NPs because of their significance in science and technology. Cr_2O_3–NPs have specific use in high-

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temperature and corrosive-resistant materials, ligand crystal displays, green pigments, catalysts, glasses, inks, paints and as precursor to the magnetic pigments (Jaswal et al. 2014). Investigations on the toxicity of Cr_2O_3–NPs are limited. Exposure of Cr_2O_3–NPs to the human lung carcinoma (A549) and human keratinocyte (HaCaT) cell lines revealed severe cytotoxicity in the cells (Horie et al. 2013). The effect of Cr_2O_3–NPs was greater than that of microparticles (MPs) after exposure to the same concentration. The NPs showed statistically significant increase in the intracellular reactive oxygen species (ROS) and activation of antioxidant defence systems in both A549 and HaCaT cell lines. Treatment with Cr_2O_3–NPs revealed activation of caspase-3, indicating that the reduction in the cell viability was due to apoptosis after exposure to NPs. It can be concluded from this study that Cr_2O_3–NPs have more cytotoxic potential compared to its MPs (Horie et al. 2013). In a study, cell viability was evaluated using environmentally relevant *Escherichia coli* bacterium; the organisms were exposed to 0–100 μg/mL Cr_2O_3–NPs for 120 min. Reduction in the cell viability was observed after the treatment (Singh et al. 2011). The acute toxicity of Cr_2O_3–NPs was assessed using *Daphnia similis*. Cr_2O_3–NPs were found to be two times more toxic than the chromium (Cr) salt, suggesting that the distinctive character of NPs which is its reduced size plays an important role in the induction of toxicity (Tavares et al. 2014). Lung exposure to metal oxide NPs may produce T helper cell type 1 (Th1)- and Th17-associated delayed-type hypersensitivity (DTH) responses and pulmonary alveolar proteinosis (PAP). Hence to investigate this effect, NPs nickel oxide (NiO), cobalt oxide, Cr_2O_3 and copper oxide (CuO) were instilled into the lungs of female Wistar rats and the immune inflammatory responses were evaluated at 24 and 4 weeks post-instillation. Primary culture of alveolar macrophages from Wistar rats was used to evaluate the effect of the NPs on the ability to clear surfactant. NiO and Cr_2O_3–NPs induced lung DTH-like responses and alveolar lipoproteinosis. However, neither Cr_2O_3 nor CuO–NPs elicited immune inflammatory reactions (Cho et al. 2012).

In vivo study of NPs is essential because animal systems are extremely complicated and the interaction of the NPs with biological models could lead to novel distribution, clearance, immune response and metabolism patterns. Moreover, oral route is an important point of entry of NPs in humans and animals. As far as we are aware, the in vivo toxicology of Cr_2O_3–NPs by oral route has not been reported till date. Therefore, the objective of the current study was to compare the genotoxicity and biodistribution of Cr_2O_3–NPs and Cr_2O_3–MPs after 28-day repeated oral treatment. It has been demonstrated that apart from the dose, toxicity of NPs is determined by their size, shape and surface area (Nel et al. 2009). Hence, in the current investigation, characterization of the test compounds was carried out by measurement of size, hydrodynamic diameter and surface charge of Cr_2O_3–NPs and MPs. Genetic toxicology is important to evaluate the hazards of NPs, as sorrow of genetic diseases and cancers is well known. Therefore, genotoxicity studies are used to get inference about the carcinogenicity potential of chemicals. The comet assay is useful for the detection of DNA damage in genotoxicity testing (Tice et al. 2000). Micronucleus test (MNT) is a promising biomarker for genotoxicity investigations. During anaphase, micronuclei (MN) are formed from chromosomal fragments or whole chromosomes that are left behind when the nucleus divides (Schmid 1975). The chromosomal aberration (CA) test can diagnose agents that cause structural chromosome or chromatid breaks, dicentrics and other abnormal chromosomes, translocations which are implicated in the various human genetic diseases and cancers (Magdolenova et al. 2014). Moreover, NPs have different patterns of absorption, distribution, elimination and biopersistence as compared with MPs due to stronger interaction of NPs with biological systems (Oberdörster et al. 2005). Hence, to gain an insight into the uptake, retention and kinetics of NPs, biodistribution studies are also mandatory.

Therefore, in the present investigation, a 28-day repeated oral dose study of Cr_2O_3–NPs and Cr_2O_3–MPs was carried in albino Wistar male and female rats. The effect of the particles on the behavioural symptoms, body weight (bw) and feed intake was examined. Genotoxicity assays such as the comet assay in peripheral blood leukocytes (PBL) and liver, MNT and CA in bone marrow cells were performed. Furthermore, biodistribution of Cr in rat's whole blood, liver, kidney, brain, heart, spleen, lung, urine and faeces was analyzed using inductively coupled plasma optical emission spectrometer (ICP-OES). The doses used to evaluate the toxicity of Cr_2O_3–NPs and Cr_2O_3–MPs were 30, 300 and 1000 mg/kg bw/day. The highest dose of 1000 mg/kg bw/day was chosen with the aim of obtaining toxic effects but not death or severe suffering in the animals. Thereafter, a descending sequence of dose levels (300 and 30 mg/kg bw/day) was selected with a view to demonstrate any dosage-related response and any no-observed-adverse effects at the lowest dose level (NOAEL) as per OECD guideline 407 (2008).

Materials and methods

Nanoparticles and chemicals

Cr_2O_3–NPs of size <40 nm and purity ≥98.4 % (according to the manufacturer's datasheet) were purchased from Mukherjee Industries Kolkata, India. Cr_2O_3–MPs of size <50 μm [CAS No. 1308-38-9] and purity ≥98 % were purchased from Sigma Chemical Co. Ltd. (St. Louis, MO, USA). Phosphate-buffered saline (Ca^{2+}, Mg^{2+} free; PBS), cyclophosphamide (CP), normal melting agarose (NMA), low melting agarose (LMA), etc. were also purchased from Sigma

Chemical Co. Ltd. (St. Louis, MO, USA). All other chemicals and plastic ware were obtained locally and were of analytical reagent grade.

Characterization of Cr_2O_3–nanoparticles and Cr_2O_3–microparticles

The particles were characterized using transmission electron microscopy (TEM), dynamic light scattering (DLS) and laser Doppler velocimetry (LDV) to evaluate the material size, size distribution, state of dispersion and zeta potential of the NPs in Milli-Q water. Characterization of Cr_2O_3–NPs and Cr_2O_3–MPs was performed to assess the size and morphology using a TEM (JEM-2100, JEOL, Japan). The images were obtained from TEM with an accelerating voltage of 120 kV. The TEM was equipped with a plunge freezer and cryotransfer holder to fix specimens in the frozen state and fitted with a Gatan 2Kx2K CCD camera for acquiring high-resolution images. Particles were suspended in Milli-Q water at a concentration of 0.01 mg/ml, and one drop of suspension was placed on a carbon-coated copper TEM grid and evaporated at room temperature. The software for advanced microscopy techniques was used for the digital TEM camera. This software was calibrated for nanoscale size measurements for the accurate examination of NPs. For the size measurement, 100 particles were calculated from random fields of view and images showing the general morphology of the particles.

The size distribution of the NPs was measured by DLS whereas zeta potential and electrophoretic mobility were measured by LDV (Fourest et al. 1994) using a Malvern Zetasizer Nano ZS (Malvern Instruments, UK). This device uses a 4-mW He–Ne 633-nm laser to analyze the samples and an electric field generator for the LDV measurements. At a concentration of 40 μg/ml, freshly prepared Cr_2O_3–NP and MP suspensions in Milli-Q water were ultrasonicated using a probe sonicator (UP100H, Hielscher Ultrasonics GmbH, Teltow, Germany) for 10 min at 90 % amplitude (100 W, frequency 30 kHz). The high concentration of the suspension was further diluted two times and adjusted to a lower concentration of 20 μg/ml to acquire enough counts per second. The prepared samples were transferred to a 1.5-ml square cuvette for DLS measurements, and 1 ml of the suspension was transferred to a Malvern Clear Zeta Potential cell for LDV measurement. The mean NP diameter was calculated using the same software program as utilized in the NP distribution, and the polydispersity index (PdI) was used to measure the size ranges present in the solution. The PdI scale ranged from 0 to 1, where 0 indicates monodisperse and 1 indicates polydisperse state of particles. The specific surface area of Cr_2O_3–NPs and Cr_2O_3–MPs was evaluated Brunauer–Emmett–Teller (BET) analysis.

Animals

Male and female albino Wistar rats, aged 6–8 weeks and weighing 80–120 g, were obtained from the National Institute of Nutrition, Hyderabad, India. The animals were acclimatized for 1 week in groups of five in polypropylene cages. The animals were fed with a standard laboratory pellet diet, and reverse osmosis water was provided ad libitum and maintained under standard conditions of humidity (55–65 %), temperature (22±3 °C) and light (12-h light/12-h dark cycles). The study was approved by the Institutional Animal Ethics Committee (IICT/BIO/TOX/PG/18/03/2011).

Treatment of animals

Twenty-eight-day repeated dose oral toxicity study was conducted with male and female rats with Cr_2O_3–NPs and Cr_2O_3–MPs based on OECD guideline 407 (2008). For the treatment, rats were weighed daily and concentrations were calculated for the doses 30, 300 and 1000 mg/kg bw for Cr_2O_3–NPs and Cr_2O_3–MPs. The rats were administered orally once daily for 28 days using a suitable intubation cannula with a dosing volume of 2 ml/100-g bw. Particles were suspended in Milli-Q water, properly ultrasonicated (UP100H, Germany) and vortexed before every treatment of the rats. Animals were divided into four groups (ten rats; five males and five females in each group): control, low dose (30 mg/kg bw/day), medium dose (300 mg/kg bw/day) and high dose (1000 mg/kg bw/day) of Cr_2O_3–NPs and Cr_2O_3–MPs. Control group received only Milli-Q water. The doses were selected based on the results of a preliminary dose range finding study (data not shown). The highest dose was selected based on induction of a toxic effect without severe sufferings and mortality, whereas the lowest dose demonstrated no-observed-adverse effects. Feed consumption and bw were monitored weekly for 4 weeks. All treated rats were sacrificed by cervical dislocation after 24 h of last administration of a dose. A known mutagen CP was used as the positive control for genotoxicity studies at a dose 40 mg/kg bw, and the volume injected was 0.01 ml/g bw. It was administered intraperitoneally (IP) 24 h before sacrifice.

Comet assay

The alkaline comet assay was used for the assessment of DNA damage in the rats after 28-day repeated oral exposure to the Cr_2O_3–NPs and Cr_2O_3–MPs. It was performed according to the method of Tice et al. (2000) with slight modifications. Whole-blood withdrawal was done in EDTA-coated tubes from retro-orbital plexus of the animals. Liver samples were collected after the sacrificing of rats. The comet assay in liver tissue was performed following the technique according to Miyamae et al. (1998). The tissue was removed from the rats after sacrificing at various time intervals, minced and

suspended at ~100 mg/ml in chilled homogenizing buffer (pH 7.5) and homogenized gently at a speed of 500–800 rpm. Cell viability was determined by the trypan blue exclusion assay (Pool-Zobel et al. 1994). Three slides were prepared for each experimental condition. In brief, microscope slides were pre-coated with 120 μl of 0.75 % NMA in PBS and allowed to solidify overnight at 37 °C after covering with coverslip for uniform layer. For second layer preparation, 10 μl of whole blood (10,000–30,000 lymphocytes) or liver homogenate was suspended in 120 μl of 0.37 % LMA. The suspension of cells and LMA was pipetted onto pre-coated slides and spread uniformly covering with coverslip. The slides were dried at 4 °C for 10 min. A third layer of plain 0.37 % LMA (120 μl) was applied, and a coverslip was quickly put to get an even layer and dried at 4 °C. After removing the coverslip, the slides were immersed in chilled lysis buffer (2.5 M NaCl, 0.1 M Na_2 EDTA, 0.2 M NaOH, 1 % Triton X-100, 10 % DMSO, pH 10.0) for 10 h at 4 °C. The slides were pre-soaked for 20 min in alkaline buffer (10 M NaOH, 200 mM Na_2 EDTA, pH >13.0), and then, electrophoresis was performed at 25 V adjusted at 300 mA for 20 min. The slides were neutralized twice in 0.4 M Tris buffer, pH 7.5, for 5 min and once in absolute methanol for 5 min. Coded slides were scored after staining with ethidium bromide (20 μg/ml) using a fluorescence microscope (Olympus, Shinjuku-ku, Tokyo, Japan) with a blue (488 nm) excitation filter and yellow (515 nm) emission (barrier) filter at ×400 magnification. A total of 150 randomly selected peripheral blood lymphocytes (PBL) and liver cells per rat (50 cells per slide) were used to measure the amount of DNA damage and expressed as percentage (%) of DNA in the comet tail. Quantification of DNA breakage was realized by using a Comet Image Analysis System, version Komet 5.5 (Single cell Gel Electrophoresis analysis company, Andor Technology 2005, Kinetic Imaging Ltd., Nottingham, UK).

Micronucleus test

The MNT in the rat bone marrow cells was carried out following the OECD Guideline 474 (1997). After 28-day repeated oral treatment, the bone marrow was removed from both the femur and tibia by aspiration into hypotonic solution of 1 % sodium citrate and centrifuged at approximately 1000 rpm for 5 min. The cell pellet was resuspended in a drop of 1 % sodium citrate, and a smear was prepared on a microscope slide and allowed to dry in humidified air overnight. The stained slides were used for the assessment of the MN occurrence. Three slides were made for each animal; the slides were microscopically analyzed at ×1000 magnification. Randomly, 2000 polychromatic erythrocytes (PCEs) per animal were selected from three slides and the frequency of micronucleated PCEs (MN-PCEs) was determined. In order to determine the ratio of PCEs to normochromatic erythrocytes (NCEs) in the bone marrow, approximately 1000 cells from each animal were examined and the ratio was expressed as percentages: (PCEs×100/PCEs+NCEs).

Chromosomal aberration assay

The method described by Adler et al. (1984) was used for CA analysis and performed in bone marrow cells. It is globally recommended to follow the OECD Guideline 475 (1997) as a test method to identify chromosomal aberrations (CAs). For analysis of metaphase cells, cell division was arrested by a mitotic inhibitor, colchicine (0.020 %), 0.01 ml/g bw IP 2 h prior to sacrifice after 28-day repeated oral treatment. The bone marrow was collected from femur and tibia bones by rinsing in hypotonic solution with 0.9 % sodium citrate centrifuged at 2000 rpm for 20 min. Cells were then fixed through several changes of ice-cold Carnoy's solution (methanol/acetic acid, 3:1 *v*/*v*) until the pellets were clean. After refrigeration for at least 24 h, cells were centrifuged and resuspended in fresh fixative, i.e. Carnoy's solution, dropped onto slides, dried and stained with Giemsa. Three slides for each animal were made by the flame-dried technique. Five hundred well-spread metaphases per dose (100/animal) were selected to detect the presence of CAs. The mitotic index (MI) was determined with 1000 or more cells.

Chromium content analysis in tissues

The biodistribution study of the Cr_2O_3–NPs and Cr_2O_3–MPs in male and female Wistar rats was carried after 28-day repeated oral treatment. The animals were placed in metabolic cages after treatment to collect the urine and faeces samples. Rats were sacrificed through cervical dislocation, and whole blood, liver, kidneys, heart, brain, lung and spleen were collected after 28 days. The samples were processed using the method of Gómez et al. (1997). The samples were pre-digested in nitric acid overnight and heated at 80 °C for 10 h, followed by additional heating at 130–150 °C for 30 min. Subsequently, a volume of 0.5 ml of 70 % per chloric acid was added, and the samples were again heated for 4 h and evaporated nearly to dryness. Following digestion, the samples were filtered, and 2 % nitric acid was added to a final volume of 5 ml for analysis. The standard solution of Cr was serially diluted to 100, 50, 10 and 1 ppm was found to get intensity of samples. The Cr content in the samples was determined using ICP-OES (JY Ultima, Jobin Yvon, France).

Statistical analysis

The statistical significant changes between treated and control groups were analyzed by one-way ANOVA. All results were expressed as mean and standard deviation (mean±SD) of five animals. Multiple pairwise comparisons were done using the

Dunnett's multiple comparison post-test to verify the significance of positive response. Statistical analyses were performed using GraphPad InStat Prism 3 Software package for Windows (GraphPad Software, Inc., La Jolla, CA, USA). The statistical significance for all tests was set at $p<0.05$.

Results

Characterization of Cr_2O_3–nanoparticles and Cr_2O_3–microparticles

The primary particle size of Cr_2O_3–NPs and Cr_2O_3–MPs was determined by TEM. The images obtained are given in Fig. 1. The mean size of Cr_2O_3–NPs and Cr_2O_3–MPs was 34.89±2.65 nm and 3.76±3.41 μm, respectively. Cr_2O_3–NPs showed 106.7-nm size with DLS measurement in Milli-Q water suspension. The DLS result with Cr_2O_3–NPs showed larger values than NP size measured by TEM, indicating that Cr_2O_3–NPs formed larger agglomerates in water suspension. Zeta potential and electrophoretic mobility measurements using LDV were 0.655 mV and 1.01 μ (μm cm/V/s), respectively, at pH 7.0. In the case of Cr_2O_3–MPs, DLS and LDV data was found to be out of detection limit. The specific surface area of Cr_2O_3–NPs and Cr_2O_3–MPs determined by BET analysis was 29.08 and 2.74 (m^2/g), respectively (Table 1).

Animal observation, food consumption, body weight and organ weight

No adverse sign, symptoms or mortality were observed after 28-day repeated oral doses of 30, 300 and 1000 mg/kg bw/day of Cr_2O_3–NPs and Cr_2O_3–MPs in male and female Wistar rats. However, NPs at medium and high doses and MPs at the highest-dose-treated rats showed dullness, irritation and moribund symptoms in the first week of study. Further, both NP- and MP-treated rats showed loss in body weight and feed intake, but the loss was not statistically significant (data not shown).

Comet assay

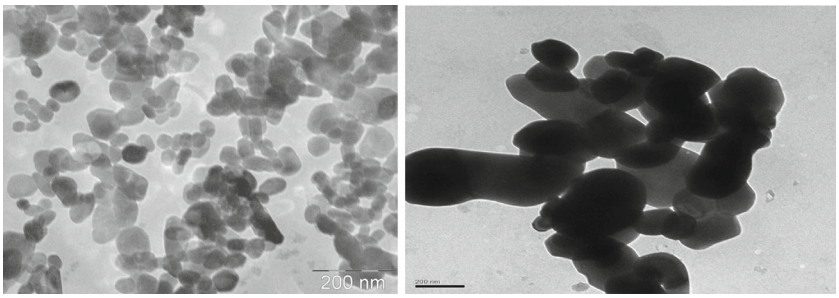
The results obtained by comet assay after 28-day repeated oral administration with 30, 300 and 1000 mg/kg bw/day doses of Cr_2O_3–NPs and Cr_2O_3–MPs to male and female Wistar rats are shown in Table 2. A statistically significant ($p<0.01$) increase in the DNA damage (% tail DNA) was observed in the PBL of rats exposed to Cr_2O_3–NPs after 28-day repeated oral doses of 300 and 1000 mg/kg bw/day in male and female Wistar rats (9.22±2.24, 12.69±1.84 and 10.24±02.46, 13.07±2.05, respectively). An enhancement in % tail DNA was also observed in the PBL at 30 mg/kg bw/day dose, but the increase was statistically insignificant after 28-day repeated dose treatment compared to control.

The % tail DNA migration calculated in the PBL of male and female rats repeatedly treated for 28 days with 1000 mg/kg bw/day of Cr_2O_3–MPs was statistically significant (10.05±1.16 and 10.95±3.12, respectively). However, Cr_2O_3–MPs at the doses of 30 and 300 mg/kg bw/day did not show significant difference ($P>0.05$) in mean % tail DNA compared to the control after 28 days of treatment.

The comet assay was performed after 28 days of repeated oral treatment with Cr_2O_3–NPs and Cr_2O_3–MPs with doses of 30, 300 and 1000 mg/kg bw/day in the liver cells of male and female rats. Statistically significant difference in % tail DNA was observed in liver cells of male and female rats treated with 300 and 1000 mg/kg bw/day doses of Cr_2O_3–NPs (11.40±2.83, 18.09±3.61 and 12.64±1.65, 21.07±4.12, respectively) in comparison to control (5.40±1.29 and 5.93±1.97). After 28 days of repeated dose treatment, 30 mg/kg bw/day dose of Cr_2O_3–NPs did not show significant increase in DNA damage in male and female rats. Cr_2O_3–MPs at 1000 mg/kg bw/day showed statistically significant ($p<0.01$) increase in % tail DNA (12.13±2.16 and 14.95±3.12, respectively) in male and female rats compared to the control group (Table 3). Nevertheless, Cr_2O_3–MPs did not elucidate statistically significant % in percent tail DNA with 30 and 300 mg/kg doses at 28 days after repeated oral exposure over control. The positive control rats showed a significantly higher mean % of tail DNA compared with control. The cell viability by the trypan blue exclusion technique was 92 % (data not shown).

Micronucleus test

The incidence of MN after treatment with Cr_2O_3–NPs and Cr_2O_3–MPs was evaluated in bone marrow cells of male and female rats after 28-day repeated oral treatment. The bone marrow MNT data revealed a statistically significant induction of MN in bone marrow cells treated with different doses of Cr_2O_3–NPs compared to control. The mean frequency of MN-PCEs in bone marrow cells of rats orally exposed to 300 and 1000 mg/kg bw/day doses of Cr_2O_3–NPs was significant (9.0±1.87, 12±2.23 and 8.4±2.6, 11.4±3.91, respectively) in male and female rats. Likewise, the mean frequency of MN-PCEs in bone marrow cells of rats treated orally with 1000 mg/kg bw/day doses of Cr_2O_3–MPs was statistically significant (9.8±2.28 and 9.4±2.86, respectively) in both sexes. However, Cr_2O_3–NPs at 30 mg/kg bw/day and Cr_2O_3–MPs at 30 and 300 mg/kg bw/day caused an enhancement in mean frequency of MN PCEs in bone marrow cells of rats in comparison to the control, but they were statistically insignificant (Table 4).



a) Cr_2O_3 - NPs b) Cr_2O_3 - MPs

Fig. 1 Characterization of Cr_2O_3–NPs and Cr_2O_3–NPs in Milli-Q water mixing done via probe sonication for 10 min. **a** Cr_2O_3–NPs. **b** Cr_2O_3–MPs

Chromosomal aberration assay

The CA assay was performed in rat bone marrow cells after administration of repeated doses of Cr_2O_3–NPs and Cr_2O_3–MPs for 28 days in male and female Wistar rats. Rat's bone marrow cells evaluated for total cytogenetic changes (numerical and structural CA) after repeated treatment for 28 days with 300 and 1000 mg/kg bw/day of Cr_2O_3–NPs (14.2±4.6, 20.6±3.2 and 15.2±5, 22.2±2.9, respectively) revealed a significant ($P< 0.01$) induction of total CAs compared to the control (3.4±1.6 and 3.0±1.7, respectively; in male and female rats). Similarly, the total cytogenetic changes calculated after 28-day repeated oral treatment with 1000 mg/kg bw/day of Cr_2O_3–MPs (15.6±2.6 and 14.2±3.8, respectively, in female and male rats) showed a significant increase in CAs ($P< 0.01$) compared to control. Nonetheless, 28-day repeated oral treatment with 30 mg/kg bw/day of Cr_2O_3–NPs and 30 and 300 mg/kg bw/day of Cr_2O_3–MPs did not reveal any significant increase in the numerical CAs or structural CAs (Tables 5 and 6)

Biodistribution of Cr_2O_3–nanoparticles and microparticles

Repeated oral exposure of Cr_2O_3–NPs and Cr_2O_3–MPs for 28 days with various doses 30, 300 and 1000 mg/kg bw/day in male and female Wistar rats was carried out by ICP-OES. The results revealed a statistically significant increase in Cr concentration, indicating that accumulation had occurred (Fig. 2). Cr content was highest in the kidneys at the experimental doses of 30, 300 and 1000 mg/kg bw/day (6.8, 12.3 and 23.12 μg/g in males and 6.9, 15.6 and 22.59 μg/g in female rats) of the Cr_2O_3–NP-treated group followed by 4.44, 7.02 and 12.12 μg/g and 4.9, 7.38 and 13.48 μg/g, respectively, in male and female rats in the Cr_2O_3–MP-treated group. The Cr content was 4.44, 8.09 and 18.87 μg/g and 4.57, 9.91 and 19.97 μg/g, respectively, in the liver of male and female rats treated with Cr_2O_3–NPs. However, Cr_2O_3–MP-treated animals at same doses showed Cr values of 2.93, 5.22 and 9.61 μg/g and 2.95, 5.63 and 9.85 μg/g, respectively. In case of spleen, the levels of Cr were 7.19, 10.46 and 17.9 μg/g and 6.84, 10.29 and 17.39 μg/g, respectively, in male and female rats, treated with the Cr_2O_3–NPs, whereas Cr_2O_3–MP-treated groups exhibited Cr quantities of 3.27±5.22 and 11.93 μg/g and 3.15, 5.52 and 11.32 μg/g, respectively, in male and female rats. Further, rats treated with Cr_2O_3–NPs showed values of 3.07, 5.34 and 10.01 μg/g and 3.02, 5.83 and 10.29 μg/g of Cr in the heart when compared to Cr_2O_3–MP-treated groups which had values of 2.09, 3.15 and 3.91 μg/g and 2.15, 3.06 and 3.61 μg/g, respectively, in male and female rats. The rats dosed with 30, 300 and 1000 mg/kg bw of Cr_2O_3–NPs showed Cr levels of 3.03, 6.40 and 9.55 μg/g and 3.08, 6.16 and 9.92 μg/g, respectively, in blood of male and female animals whereas Cr_2O_3–MP-treated animals exhibited Cr values of 2.13, 3.48 and 5.12 μg/g and 2.32, 3.65 and 5.29 μg/g, respectively, in male and female rats. After the treatment with Cr_2O_3–NPs, the lungs of rats exhibited Cr quantities of 1.21, 3.08 and 5.53 μg/g and 1.27, 3.18 and 5.3 μg/g, respectively, in male and female rats when compared to 1.08, 1.32 and 1.95 μg/g and 1.05, 1.46 and 1.99 μg/g, respectively, in male

Table 1 Characterization of Cr_2O_3–NPs and Cr_2O_3–MPs

Particles	Size using TEM (nm)	DLS		LDV			Surface area (m^2/g)
		Average diameter (nm)	PdI	Zeta potential ζ (mV)	Electrophoretic mobility (μm cm/s V)	pH	
Cr_2O_3–NPs	34.89±2.65	106.7±11.21	0.304	0.655	−1.15	7.0	19.08
Cr_2O_3 –MPs	3.76±3.41	ND	ND	ND	ND	7.0	1.74

PdI polydispersity index, *DLS* dynamic light scattering, *LDV* laser Doppler velocimetry, *ND* not detectable

Table 2 Mean % tail DNA in PBL of male and female Wistar rats observed after 28-day repeated oral doses of Cr_2O_3–NPs and Cr_2O_3–MPs

Treatments	Dose (mg/kg bw)	% Tail DNA	
		Male	Female
Control	Distilled water	4.70±0.79	4.94±0.87
Cr_2O_3–NPs	30	5.87±1.12	6.03±1.05
	300	9.22±2.24*	10.24±02.46*
	1000	12.69±1.84*	13.07±2.05*
Cr_2O_3–MPs	30	6.29±1.01	6.36±1.82
	300	8.02±1.42	8.56±2.24
	1000	10.05±1.16*	10.95±3.12*
CP	40	46.89±8.34*	43.67±5.78*

Data represented as mean±SD. The statistical significance for all tests was set at $p<0.05$

Significantly different from control at $*p<0.01$, $n=5$ animals per group

and female animals in Cr_2O_3–MP-treated group. Lowest amount of Cr was found in brain (1.55, 2.01 and 2.6 μg/g and 1.59, 2.1 and 2.72 μg/g, respectively, in male and female rats) of animals treated with Cr_2O_3–NPs followed by 1.01, 1.32 and 1.71 μg/g and 1.05, 1.21 and 1.90 μg/g, respectively, in male and female rats in animals treated with Cr_2O_3–MPs. The maximum content of Cr was excreted in the faeces in Cr_2O_3–MP-treated group followed by Cr_2O_3–NP-treated rats in a dose-dependent manner.

Discussion

It has been reported that at least 1000 consumer goods containing NPs are available commercially (Tomankova et al. 2015). Human exposure to NPs is already occurring and will increase significantly in the future. Although no human illnesses to date are confirmed to be attributed to NPs, toxicity studies are needed to verify the health effects of engineered NPs. The intake of NPs by the oral route has the chance for wider exposure of the public to higher doses and more frequent ingestion in comparison to inhalation or skin exposure (Rashidi and Khosravi-Darani 2011). However, the data reported for NP exposure via oral route is scare. Genotoxicity assessment was carried out by comet assay, MNT and CA assay in male and female rats after 28-day repeated oral treatment with 30, 300 and 1000 mg/kg bw/day of Cr_2O_3–NPs and MPs. Genotoxicity investigations performed using comet assay, MNT and CA showed that Cr_2O_3–NPs were able to cause significant enhancement in % tail DNA in liver, MN formation and CA in bone marrow cells at 300 and 1000 mg/kg bw /day dose in comparison to control. However, the Cr_2O_3–MPs revealed significant effect only at 1000 mg/kg bw/day. Similar investigations with Cr_2O_3–NPs are not available in literature yet. However, some studies with other NPs are in agreement with our findings. When mice were given 300 mg/kg bw/day zinc oxide NPs by oral route for 14 continuous days, significant increase in DNA damage with comet assay in the liver at the higher dose was found (Sharma et al. 2012). Manganese oxide NPs administered orally with 300 and 1000 mg/kg/bw for 28 days induced DNA damage in PBL and liver with comet assay in rats (Singh et al. 2013). The results from our investigations suggest that clastogenic events may be involved in the formation of MN with Cr_2O_3–NPs. Our findings are in concurrence with a study in mice. Significant increase in the MN frequency was observed in PCEs, when 500 mg/kg/bw of titanium oxide (TiO_2)–NPs were administered in drinking water for 5 days (Trouiller et al. 2009). On the contrary, when C57BL/6J mice were exposed to TiO_2–NPs by inhalation route for 5 days (4 h/day) at 0.8, 7.2 and 28.5 mg/m^3 and MN were analyzed by acridine orange staining in blood PCEs collected 48 h after the last exposure, insignificant effect on the level of DNA damage in lung epithelial cells or MN in PCEs was observed, suggesting no genotoxic effects by the 5-day inhalation exposure to nanosized TiO_2 (Lindberg et al. 2012).

Our results showed a decrease in MI value in Cr_2O_3–NPs and MPs compared to control groups. This could be due to a slower progression of cells from S (DNA synthesis) phase to M (mitosis) phase of cell cycle as a result of NP and MP exposure. Although it is most likely that this impairment in cell cycle progression is associated with NPs and MPs toxicity. Our CA assay results are in agreement with an investigation on Cerium oxide (CeO_2)–NPs. The NPs were administered orally to rats for 28 days continuously at the doses of 30, 300 and 600 mg/kg/day. Significant increase was observed in the frequency of CA in the bone marrow cells (Kumari et al. 2014).

The biodistribution study revealed that Cr ions from NP- or MP-treated rats could pass across the gastrointestinal barrier and accumulate in the organs and tissues. Significantly

Table 3 Mean % tail DNA in liver cells of male and female Wistar rats observed after 28-day repeated oral doses of Cr_2O_3–NPs and Cr_2O_3–MPs

Treatments	Dose (mg/kg bw)	% Tail DNA	
		Male	Female
Control	Distilled water	5.40±1.29	5.93±1.97
Cr_2O_3–NPs	30	7.07±2.08	7.43±1.52
	300	11.40±2.83*	12.64±1.65*
	1000	18.09±3.61*	21.07±4.12*
Cr_2O_3–MPs	30	6.89±1.58	6.83±1.67
	300	7.21±1.32	7.37±1.34
	1000	12.13±2.16*	14.95±3.12*
CP	40	49±8.57*	44±5.89*

Data represented as mean±SD. The statistical significance for all tests was set at $p<0.05$

Significantly different from control at $*p<0.01$, $n=5$ animals per group

Table 4 Frequency of MN-PCEs and % PCEs in male and female Wistar rat bone marrow cells treated orally after 28-day repeated oral exposure of Cr_2O_3–NPs and Cr_2O_3–MPs

Treatments	Dose (mg/kg bw)	Male		Female	
		MN-PCEs	% PCEs	MN-PCEs	% PCEs
Control	Distilled water	2.8±0.83	42.98	2.4±1.14	43.34
Cr_2O_3–NPs	30	5.8±1.92	42.79	4.6±1.41	43.13
	300	9.0±1.87*	42.04	8.4±2.60*	42.00
	1000	12±2.23*	41.30	11.4±3.91*	41.00
Cr_2O_3–MPs	30	5.0±1.41	42.58	4.4±1.67	42.89
	300	5.97±2.14	6.13±2.11	6.13±2.11	42.24
	1000	9.8±2.28 *	41.81	9.4±2.86*	41.36
CP	40	33.2±3.13**	31.40	36.6±3.66**	32.03

Data represented as mean±SD. Multiple comparisons were performed by Dunnett's test. The statistical significance for all tests was set at $p<0.05$

Significantly different from control at *$p< 0.01$, **$p<0.001$, $n=5$ animals per group

increased Cr levels were detected in organs such as liver, spleen, kidney, heart, blood, brain and lungs of rats orally exposed with Cr_2O_3–NPs. The majority of Cr content from NP-treated rats was found in the kidney, liver and spleen. The excretion data showed that a little quantity of Cr was excreted via urine, whereas large amounts of the Cr ions were excreted via faeces. The tissue distribution of Cr_2O_3 MP-treated rats also showed significant increase in higher dose, and some amount was observed in the kidney, heart, spleen, lungs and blood with medium and low doses. Our study indicated that in comparison to the Cr levels from NP-treated rats, less amount of Cr content from MP-treated rats can pass through the intestinal barrier and large amounts of Cr ions were quickly excreted in faeces. The excretion of the Cr ions was greater in faeces of MP-treated rats in contrast to NP-exposed animals. The distribution pattern of Cr content from NP-treated rats was dose dependent. The amount of Cr ions absorbed increased as the dose administered was raised. Moreover, an insignificant gender-related difference in the biodistribution of Cr ions was found in the different tissues of male and female Wistar rats during 28-day repeated oral dose study. The Cr level was slightly higher in brains of the NP-treated animals when compared to MPs as well as control groups. This study indicated that Cr ions from NP-treated rats can penetrate the blood–brain barrier (BBB). Similarly, Kim et al. (2006) detected magnetic NPs in brain with doses of 100, 50 and 25 mg/kg of silica-overcoated magnetic NPs containing rhodamine B isothiocyanate when administered to the mice intraperitoneally for 4 weeks, indicating that nanosized materials can penetrate BBB. Cr ion accumulation was highest in the kidneys from the Cr_2O_3–NP-treated animals followed by the liver and spleen of male and female rats. Similar biodistribution investigations with Cr_2O_3–NPs and MPs by oral route have not been reported yet. Some studies with other NPs are also in concurrence of our findings. The toxicity of silicon dioxide (SiO_2), silver (Ag) and iron oxide (Fe_2O_3) NPs after repeated oral administration for 13 weeks to Sprague–Dawley rats was evaluated. The highest dose selected was 975.9, 1030.5 and 1000 mg/kg of the SiO_2, Ag and Fe_2O_3 NPs, respectively. SiO_2 and Fe_2O_3–NPs did not show systemic distribution in blood, organs, urine and faeces tested, whereas the Ag–NP-treated group revealed significant dose-related increase. The authors suggested that the systemic toxicity of the Ag–NPs, including liver and kidney toxicity, might be explained by extensive systemic distribution of Ag originating from the Ag–NPs (Yun et al. 2015). The toxicity and biodistribution of aluminium oxide (Al_2O_3) NPs after a 13-week repeated dose oral administration with 1.5, 3 and 6 mg/kg dose in mice were studied. The Al_2O_3–NP-treated animals showed the highest accumulation of aluminium (Al) in the liver and kidneys compared with the control group (Park et al. 2015). In a study, Wistar rats were treated with Al_2O_3–NPs of different sizes (30 and 40 nm) by oral route at the doses of 2000, 1000 and 500 mg/kg bw/day. The results revealed dose- and size-dependent accumulation of Al content in the different organs viz. whole blood, liver, heart, kidney, brain and spleen of rats after 14 days of treatment. The bulk counterparts of the same materials did not show significant increase of Al in various tissues and were mainly excreted by the faeces (Balasubramanyam et al. 2009). An in vivo biodistribution study with silicon carbide (SiC) NPs after sub-acute (28 days) oral treatment in rats was carried out by Particle-Induced X-ray Emission technique. For the study, sub-acute doses of 0.5 and 50 mg/kg bw/day were selected. The result of this study showed that significant increase of SiC was not observed in the different organs, and most of the NPs were cleared by the faeces, and low traces were excreted by the urine (Lozano et al. 2012). Likewise, tissue distribution study of CeO_2–NPs and MPs was carried out in rats after 28-day repeated exposure with 30, 300 and 600 mg/kg bw/kg doses by oral route. The results revealed that a significant accumulation of the cerium (Ce) concentration in whole blood, liver,

Table 5 Chromosome aberrations and percent mitotic index observed in bone marrow cells of female Wistar rats after 28-day repeated oral treatment with Cr_2O_3–NPs and Cr_2O_3–MPs

Dose (mg/kg bw)	M.I. (%) *M*±SE	Chromosomal aberrations							Aberrant cells (%)	Total cytogenetic changes	TA + gaps *M*±SE	TA − gaps *M*±SE
		Numerical aberrations		Structural aberrations								
		Aneuploidy	Polyploidy	Gaps	Breaks	Minute	AF	RT				
Con.	3.25±0.24	1.0±0.7	0.0±0.0	1.0±0.5	0.4±0.5	0.2±0.4	0.4±0.4	0.00±0	0.6±0.24	3.0±1.7	2.0±1.2	1.0±0.7
Cr_2O_3NPs												
30	2.73±0.17	1.6±0.5	0.0±0.0	1.4±0.9	1.2±0.8	1.0±0.7	0.8±0.8	0.0±00	2.9±0.80	6.0±1.9	4.4±1.5	3.0±0.1
300	2.89±0.21	4.4±1.9	0.0±0.0	3.6±1.9	3.0±1.6	2.0±0.8	2.2±1.6	0.0±00	7.2±2.4*	15.2±5*	10.8±3.9*	7.2±3.03*
1000	2.74±0.17	7.6±2.6	0.0±0.0	5.8±2.6	3.8±1.9	2.4±1.7	2.6±2.07	0.0±00	13.1±3.7*	22.2±2.9*	14.6±4.67*	8.8±4.1*
Cr_2O_3–MPs												
30	3.10±0.19	2.0±0.7	0.0±0.0	1.4±0.9	1.0±0.7	0.6±0.5	0.6±0.5	0.0±0.0	2.3±0.79	5.6±1.8	3.6±1.7	2.2±0.8
300	2.81±0.15	3.8±2.16	0.0±0.0	2.0±1.4	1.8±0.9	1.2±0.8	0.1±0.7	0.0±0.0	4.2±2.0	6.10±1.12	5.14±1.71	3.12±2.3
1000	2.93±0.31	4.6±2.7	0.0±0.0	4.2±1.3	2.6±1.14	1.8±0.8	2.4±1.17	0.0±0.0	10.4±3.4*	15.6±2.6*	11.±3.07*	6.8±2.6*
CP	1.98±0.28	38.4±1.8**	3.4±0.5**	12.2±1.6**	10.4±1.3**	11.6±1.9**	13±1.2**	1.8±0.5**	35±6.1**	90.8±5.4**	49±3.61**	36.8±3.51**

Data represented as mean±SD. One hundred metaphases were analyzed per animal; $n=5$ animals per group. Total cytogenetic changes = numerical aberrations and structural aberrations. % Aberrant cells correspond to cells with ≥1 aberration excluding gaps. Data represented as mean±standard error. Negative control—deionized water. Cyclophosphamide (CP=40 mg/kg). The statistical significance for all tests was set at $p<0.05$

MI mitotic index, *TA* total aberrations = structural aberrations, *AF* acentric fragments, *RT* reciprocal translations

Significantly different from control at *$p<0.01$, **$p<0.001$

kidneys, heart, brain, lung, spleen, urine and faeces was in a dose-dependent manner. Further, significant Ce content was excreted via urine in NP-treated rats whereas MP-treated rats showed more excretion in faeces probably due to their large size (Kumari et al. 2014). Similarly, an in vivo biological distribution of nanoceria NP was carried out by dosing animals weekly for 2 or 5 weeks with 0.5 mg/kg bw/day nanoceria administered to mice by different routes such as per orally (PO), intravenously (IV) or IP. Organ deposition for IV and IP mice was greatest in the spleen followed by the liver, lungs and kidneys. The results showed that the most extensive and cumulative metal deposition was via IV and IP administration while PO route showed greater excretion (more than 95 %) of nanoceria within 24 h. Elimination of Ce with all administration routes was statistically significant through faeces (Hirst et al. 2013).

Our results showed that the toxicology of Cr_2O_3–NPs was size dependent. The size of particles has been proven to be very influential to their behaviour. Accordingly, smaller particles have a wider tissue distribution, penetrate further within

Table 6 Chromosome aberrations and percent mitotic index observed in bone marrow cells of male Wistar rats after 28-day repeated oral treatment with Cr_2O_3–NPs and Cr_2O_3–MPs

Dose (mg/kg bw)	M.I. (%) *M*±SE	Chromosomal aberrations							Aberrant cells (%)	Total cytogenetic changes	TA + gaps *M*±SE	TA − gaps *M*±SE
		Numerical aberrations		Structural aberrations								
		Aneuploidy	Polyploidy	Gaps	Breaks	Minute	AF	RT				
Con.	3.15±0.11	1.2±0.7	0.0±0.0	0.8±0.4	0.6±0.4	0.4±0.5	0.4±0.4	0.00±0	0.8±0.52	3.4±1.6	2.2±1.1	1.4±0.8
Cr_2O_3–NPs												
30	2.87±0.16	2.0±0.7	0.0±0.0	1.2±0.4	0.8±0.4	0.6±0.5	0.6±0.5	0.0±00	2.7±0.43	5.2±1.3	3.2±1.3	2.0±1.0
300	2.74±0.21	5.0±1.6	0.0±0.0	4.0±1.6	2.0±0.7	1.6±0.9	1.6±1.6	0.0±00	5.9±1.7	14.2±4.6*	9.0±2.9*	5.0±1.3*
1000	2.62±0.17	6.6±3.2	0.0±0.0	5.2±2.9	3.2±1.4	2.8±1.3	2.8±1.7	0.0±00	11.8±2.9	20.6±3.2*	14±3.9*	8.8±3.4*
Cr_2O_3- MPs												
30	3.05±0.15	2.0±1.0	0.0±0.0	1.2±0.8	0.8±0.4	0.8±0.5	1.0±0.7	0.0±0.0	2.5±0.14	5.8±1.6	3.8±1.3	2.6±1.14
300	2.90±0.20	3.8±1.9	0.0±0.0	1.8±0.9	1.6±0.8	1.4±0.8	1.8±0.6	0.0±0.0	4.1±1.5	6.74±3.41	4.21±1.71	4.3±2.7
1000	2.73±0.21	5.0±2.7	0.0±0.0	3.0±1.2	2.4±1.1	1.8±0.8	2.0±1.17	0.0±0.0	9.4±2.3*	14.2±3.8*	9.2±2.9*	6.2±3.6*
CP	2.12±0.18	38.4±1.8**	3.4±0.5**	12.2±1.6**	10.4±1.3**	11.6±1.9**	13±1.2**	1.8±0.5**	32±5.6**	90.8±5.4**	49±3.61**	36.8±3.51**

Data represented as mean±SD. One hundred metaphases were analyzed per animal; $n=5$ animals per group. Total cytogenetic changes = numerical aberrations and structural aberrations. % Aberrant cells correspond to cells with ≥1 aberration excluding gaps. Data represented as mean±standard error. Negative control—deionized water. Cyclophosphamide (CP=40 mg/kg). The statistical significance for all tests was set at $p<0.05$

MI mitotic index, *TA* total aberrations = structural aberrations. *AF* acentric fragments, *RT* reciprocal translations

Significantly different from control at *$p<0.01$, **$p<0.001$

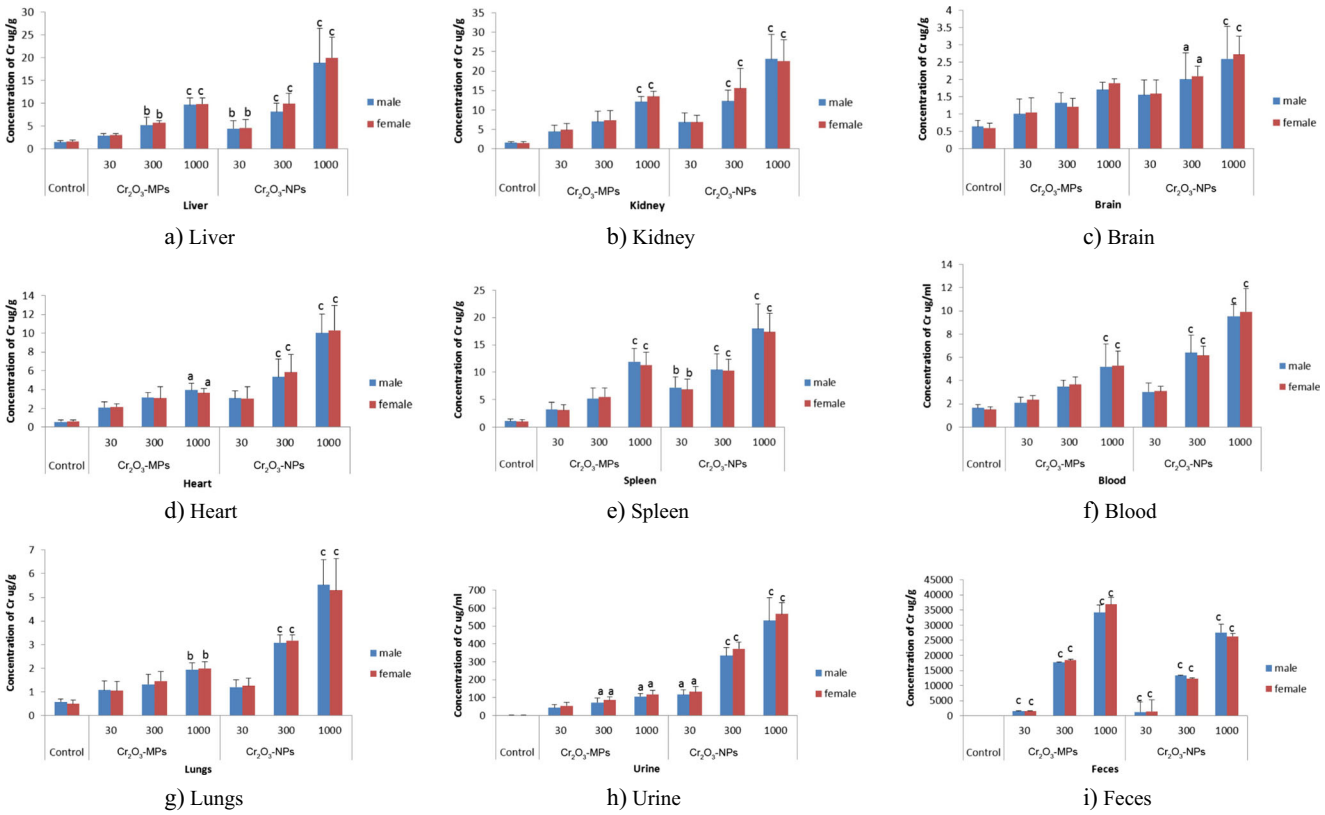


Fig. 2 Tissue distribution of Cr measured by ICP-MS in male and female Wistar rats after 28-day repeated oral doses of Cr_2O_3–NPs and Cr_2O_3–BPs at the doses of 30, 300 and 1000 mg/kg/day: **a** liver, **b** kidney, **c** brain, **d** heart, **e** spleen, **f** blood, **g** lungs, **h** urine and **i** faeces. Significantly different from control at $^{a}p<0.05$, $^{b}p<0.01$, $^{c}p<0.001$

the body of the animals, are internalized to a greater extent and have a larger toxic potency. The novel properties associated with smaller particles not only drive the exploitation of NPs but also fuel the skepticism surrounding their exploitation, due to their apparently greater toxicity than their larger counterparts. However, limited studies have directly compared NPs and MPs of metal oxides, and often, comparisons are made between different particles that all have nanodimensions. Therefore, the inclusion of a microparticulate control is of benefit to investigate, in order to decipher if effects are driven by the small size of NPs or another characteristic. In addition, it is relevant that perhaps, the toxicity of smaller Cr_2O_3–NPs is a consequence of their greater capacity to release Cr ions, which mediate the observed toxicity, but this is uncertain at this time. Furthermore, wide-ranging doses or different dose metrics have been utilized by different experiments, and so, it may not only be the size of particles that contributes to the responses observed but also the experimental set-up. However, it has been repeatedly demonstrated that NP toxicity is driven by their size, so that when administered at an equal-mass dose, smaller particles have a larger surface area and therefore exhibit a greater toxic potential.

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