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RESEARCH ARTICLE

Toxicological assessment of tungsten oxide nanoparticles in rats after acute oral exposure

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Abstract Advances in and the rapid growth of the nanotechnology sector have escalated manufacture of nanoparticles (NPs), resulting in a significant increase in the probability of exposure of humans and wildlife to these materials. Many NPs have been found to exert genotoxicity. Therefore, genotoxicity studies are mandatory to assess the toxicity of NPs as a concern of succumbing to genetic diseases and cancers are universal. Tungsten oxide (WO_3) NPs are being explored extensively in various fields. However, the toxicological data of WO_3 NPs by oral route in mammals is limited. Hence, the goal of the current investigation was to evaluate the acute toxicity of WO_3 NPs and microparticles (MPs) after single oral administration with 100, 500 and 1000 mg/kg body weight doses in female Wistar rats. TEM, dynamic light scattering and laser Doppler velocimetry techniques were used to characterise the particles. The genotoxicity studies were conducted using comet, micronucleus and chromosomal aberration assays. Alterations in biochemical indices and metal distribution in various organs were also evaluated. The mean size of WO_3 NPs and MPs by TEM was 53.2 ± 1.91 nm and 5.17 ± 3.18 μm, respectively. The results revealed a significant increase in DNA damage and micronuclei and chromosomal aberrations after exposure to 1000 mg/kg dose of WO_3 NPs. Significant alterations in aspartate transaminase, alanine transaminase, reduced glutathione, catalase and malondialdehyde levels in serum and liver were found only at the higher dose of WO_3 NPs. Tungsten (W) biodistribution was observed in all the tissues in a dose-, time- and organ-dependent manner. In addition, the maximum concentration of W was found in the liver and the least in the brain was observed. The test substances were found to have a relatively low acute toxicity hazard. The data obtained gives preliminary information on the potential toxicity of WO_3 NPs and MPs.

Keywords Tungsten oxide nanoparticles · Characterisation · Rats · Genotoxicity · Biochemical · Biodistribution

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Introduction

Nanotechnology is being recognised as the technology with the most potential to bring about a global revolution for applications in both domestic and commercial sectors through the introduction of a unique class of materials called nanoparticles (NPs). NPs are defined as materials having sizes less than 100 nm in diameter. At least a thousand products containing NPs are available on the global market (Tomankova et al. 2015). NPs have novel physiochemical properties because of their small size, which may pose a health risk to humans and wildlife as these substances can interact with cells and organs by various mechanisms (Nel et al. 2009). Therefore,

increasing concerns about their toxicity and potential environmental impact are being raised, especially as many NPs are new and untested for their toxicological properties. For example, there is evidence that suggests that carbon NPs can cause oxidative stress in cells of the fish brain and inflammation in the pulmonary region of rats (Oberdörster 2004; Warheit et al. 2004). Exposure to nanosilver (Ag) through the intravenous (IV) and intratracheal instillation in rats led to enhanced platelet aggregation and fostered the development of venous thrombus (Jun et al. 2011). Mice exposed via intraperitoneal (IP) route to silica nanocrystals (50 mg/kg) had an increase in DNA comet formation both in bone marrow and brain cells (Durnev et al. 2010). Liu et al. (2013) showed respiratory tract immune toxicity of Ag, zinc oxide (ZnO) and titanium dioxide (TiO_2) NPs in rat and alveolar macrophages. ZnO NPs induced greater cytotoxicity in comparison to Ag and TiO_2 NPs. Hepatotoxicity was explored in BRL-3A cells and Sprague-Dawley rats with nanosized TiO_2 NPs. The results suggested that NPs increased hepatotoxicity through induction of oxidative stress (Sha et al. 2014). Arai et al. (2003) studied the difference in toxicity mechanism between ion and NP forms of Ag in mouse lung and macrophages. The authors suggested that Ag NPs were transported to lysosomes and only gradually dissolved in macrophages leading to slight inflammatory stimulation in the lungs of the mouse when compared to silver nitrate. The mechanism of nanocopper (Cu)-induced nephrotoxicity was studied by analysing renal gene expression profiles phenotypically along with conventional toxicological investigations. The data revealed that Cu NPs caused proximal tubule necrosis in the rat kidneys apart from an increase in blood urea nitrogen and creatine levels (Liao and Liu 2012).

Hence, there is urgent need to assess the safety of NPs and identify their toxicological properties. Among the various manufactured NPs, tungsten oxide (WO_3) NPs have important applications in a variety of fields. WO_3 NPs have been used to construct flat panel displays; optical modulation products; optical devices having the ability to read, write and erase; photochromic smart windows; gas sensors; biomedical sensors; and humidity and temperature sensors (Baeck et al. 2003; Lu et al. 2006). WO_3 NPs are also used extensively in biological products such as pigments, additives and analytical agents (Syed et al. 2010). Portable acetone sensors made up of 10 mol% Si-doped WO_3 NPs have been developed and tested for breath analysis (Righettoni et al. 2012). Further, WO_3 NPs are being used for photocopiers, facsimile machines, laser printers, anti-static coatings, magnetrons for microwave ovens and electronic tubes (Zhou et al. 2012).

Although WO_3 NPs is very important commercially and is widely used, few studies have investigated the potential adverse effects resulting from exposure to the WO_3 NPs. WO_3 NPs revealed a positive mutagenic effect with *Salmonella typhimurium* strains TA1537 and TA 98 whereas their microparticles (MPs) showed a negative response (Hasegawa et al. 2012). WO_3 NPs evaluated using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay, in cultured rat primary hepatocytes, showed a significant decrease in cell viability at 300, 500 and 1000 ppm. However, WO_3 NPs did not cause a significant increase of micronucleated hepatocytes but increased 8-oxo-2-deoxy guanosine levels (Turkez et al. 2014). The cellular viability of 11 metal oxide NPs including WO_3 was studied against human lung adenocarcinoma (A549), human epithelial colorectal adenocarcinoma (Caco-2) and mouse embryo fibroblast (3T3) cells using neutral red uptake assay. WO_3 NPs showed toxic effects above 100 μg/ml (Ivask et al. 2015). The genotoxic potential of WO_3 NPs was examined in Sprague-Dawley rat bone marrow cells. The doses (25, 50 and 100 mg/kg body weight (bw)) of the NPs were given by IP injection for 30 days. A significant increase in MN formation was observed at 50 and 100 mg/kg bw doses; however, no increase in the incidence of chromosomal aberration (CA) was found at any of the doses (Turkez et al. 2013). Limited toxicological data on WO_3 NPs makes it difficult to determine if there is a potential toxicological risk associated with these NPs. There are no reports on the toxicity of WO_3 NPs and their MPs on Wistar rats through the oral route. The intake of NPs by the oral route has the chance for wider exposure to the public and more frequent ingestion in comparison to inhalation or dermal exposure (Rashidi and Khosravi-Darani 2011). Utilisation of NPs has increased tremendously in both consumer and manufacturing sectors in the last decade, which may result in an increased risk of exposure to humans and wildlife to NPs (Grillo et al. 2015). Thus, studying the toxicity of NPs is of importance. Further, the toxicological study of NPs in vivo is essential because animal models are more complex than in vitro models and the intercommunication of NPs with biological systems could lead to unique distribution and clearance patterns. Moreover, the oral exposure route is an important portal of entry of NPs into humans and animals. The gastrointestinal tract constitutes a primary avenue for NPs as food and water consumed by humans may contain significant concentrations of NPs. NPs may get access to the human body by adsorption from the digestive tract into the circulatory system (Volkheimer 1974). It is assumed that NPs are ingested by the M-cells of Peyer's patches, the isolated follicles of lymphoid tissues and enterocytes (Florence 1997). Hence, the oral route appears to represent one of the most important uptake routes for NPs and thus also represents an appropriate method of treatment for evaluation of NP toxicity. Though the amount WO_3 NPs being produced is not known, the world production of tungsten (W) for 2014 was 82,400 t according to Mineral commodity summaries 2015 (US Geological Survey 2015). There is evidence that besides the dose, toxicology of

NPs is decided by their surface area, size and shape (Nel et al. 2009). Hence, in the current investigation, the WO_3 NPs and MPs were characterised by using dynamic light scattering (DLS), laser Doppler velocimetry (LDV) and transmission electron microscopy (TEM) before conducting an acute oral toxicity. The acute oral toxicity study (dose-range finding study) consisted of a sighting and the main study. Signs, symptoms and mortality were documented. Changes in bw, in feed intake and in histopathology of tissues were recorded in the treated rats. The main experiment comprised the assessment of genotoxic potential, biochemical alterations and biodistribution patterns. The genotoxicity was studied using comet, micronucleus (MN) and chromosomal aberration (CA) assays. Biochemical estimations such as aspartate aminotransferase (AST) and alanine aminotransferase (ALT), reduced glutathione (GSH) content, catalase and lipid peroxidation were performed. Furthermore, biodistribution of W in rat's whole blood, urine, faeces and tissues was analysed using inductively coupled plasma optical emission spectroscopy (ICP-OES) after acute oral treatment.

Materials and methods

Nanoparticles and chemicals

WO_3 NPs (WO_3 <100 nm, 99.8%, CAS No. 1314-35-8) and WO_3 MPs (WO_3 <20 μm, 99%, CAS No. 1314-35-8) (according to the manufacture's data sheet) were purchased from Sigma Chemical Co. Ltd. (St. Louis, MO, USA). Cyclophosphamide (CP), low melting agarose (LMA), normal melting agarose (NMA) and phosphate-buffered saline (Ca^{2+}, Mg^{2+} free; PBS) were also obtained from Sigma Chemical Co. Ltd. All other chemicals of analytical grade and glassware were procured from local vendors of Hyderabad.

Characterisation of tungsten oxide nanoparticles and microparticles

The material size, distribution, zeta potential and the state of dispersion of WO_3 NPs and MPs in Milli-Q water by using various physiochemical techniques such as TEM, DLS and LDV were obtained (Singh et al. 2016). The size and morphological analysis of WO_3 NPs and MPs were evaluated by using a TEM (JEM-2100, JEOL, Japan). Hundred particles were examined from different fields of view for size measurement. The hydrodynamic size of the NPs and agglomerates was calculated through DLS and LDV with the help of Malvern Zetasizer Nano-ZS (Malvern Instruments, UK).

Animals

All procedures of the study were reviewed and approved by the Institutional Animal Ethics Committee (IICT//TOX/PG/25/06/2014/01). The rats (female albino Wistar), about 2 months old with weights ranging from 80 to 120 g, were procured from the National Institute of Nutrition, Hyderabad, India. Five animals were kept per cage (polypropylene) and acclimatised for a week. They were given a standard laboratory pellet diet and unlimited water. They were housed under standard conditions of temperature at 22 ± 3 °C, 45–65% humidity and automated light cycles (12-h light/12-h dark).

Acute oral toxicity study

The acute oral toxicity study of WO_3 NPs and MPs was assessed in accordance with OECD guideline 420 (OECD 2001). Initially, a dose-range finding study was performed with WO_3 NPs and MPs using single doses of 5, 50, 300 and 2000 mg/kg bw. The food was withdrawn from the rats 12 h before dosing. No obvious toxic signs nor mortality was observed at any of the four doses in the range finding study. Since there is little information about the toxicity of the test material, a limit test was performed by oral administration of the WO_3 NPs and MPs to four female rats with a single dose of 2000 mg/kg bw. The test animals were observed for 14 days after dosing. The feed intake and bw were tracked every day throughout the observation period. Mortality and, if any, adverse signs and symptoms were documented two times on the day of dosing and daily once during the test period. At termination, the surviving animals were weighed and sacrificed for histopathological study. After necropsy, tissues (brain, heart, liver, kidneys and spleen) were cleaned with saline and fixed in 10% formalin. The tissues were processed and then embedded into paraffin blocks. The 3-μm-thick sections obtained after slicing the blocks were mounted on a slide. The slides were stained with haematoxylin and eosin (H & E) and scrutinised with a microscope at ×400 magnification (Nikon Eclipse E 800, Japan). Three random slides per tissue were examined for histopathological damage.

Main experiment: genotoxicity, biochemical and biodistribution studies

The rats were randomly distributed into three batches consisting of a positive control (for the genotoxicity studies), negative control and experimental batch. For each batch, five animals were used. The experimental batch was further divided into three subgroups on the basis of acute toxicity data. Three doses of WO_3 NPs and MPs (100, 500 and 1000 mg/kg bw) were obtained by suspending them in Milli-Q water after ultrasonication using a probe sonicator (10 min at 90% amplitude). The higher dose of 1000 mg/kg bw was chosen to

see the toxic effect but not death or severe suffering in rats. After that, a descending sequence of dose levels (500 and 100 mg/kg bw) was selected with a view to demonstrating any dosage-related response and any no-observed-adverse-effect level (NOAEL) at the lowest dose level as per OECD guideline 420 (OECD 2001) as it allows to establish threshold toxicity values. The doses were given through oral gavage to determine the genotoxicity, biochemical parameters and biodistribution. Of Milli-Q water, 2 ml/kg bw was given to the control animals. Cyclophosphamide (CP), a known mutagen, was used as positive control at a dose of 40 mg/kg bw for genotoxicity studies. The volume (positive control) was suspended up to 1 ml in Milli-Q water and injected by IP route to the rats. It is acceptable that the positive control may be administered by a route different from the test substance and sampled at only a single time, as long as all animals are treated during the same time period. Further, untreated concurrent negative IP control need not be included if historical control data demonstrated that no deleterious or genotoxic effects are induced by the chosen solvent or vehicle. Our unpublished data revealed that Milli-Q water had no genotoxic effects; hence, we have not included negative IP control in the current study. All batches had five animals per test per sampling time. All rats received a single oral dose and were sacrificed by cervical dislocation at respective sampling times. For genotoxicity assays, the sampling times were based on OECD guidelines 489, 474 and 475 (OECD 2016; OECD 1997a, 1997b). Samples were harvested at 24 and 72 h post-treatment for biochemical parameters. For biodistribution studies, the samples were collected after 6, 24, 48 and 72 h post-treatment.

Comet assay

The alkaline comet assay was utilised for the evaluation of DNA damage in the rats after acute oral exposure to 100, 500 and 1000 mg/kg bw doses of WO_3 NPs and MPs. Whole blood and liver samples were collected after 6, 24, 48 and 72 h after dosing. The assay was performed as per the method described by Tice et al. (2000) and OECD guideline 489 (OECD 2016). Whole blood was withdrawn in EDTA-coated tubes from retro-orbital plexus. Liver samples were collected after the sacrificing of rats. The comet assay in liver was conducted according to Miyamae et al. (1998). The liver tissues were minced and suspended in 100 mg/ml chilled homogenising buffer (pH 7.5) and homogenised gently at 400–600 rpm with Miccra D-1 high-speed tissue homogeniser. Cell viability was determined by the trypan blue exclusion assay (Pool-Zobel et al. 1994). Cells from blood and liver homogenate were diluted with PBS separately and mixed with 0.4% trypan blue dye and were counted using a haemocytometer. Three slides were prepared for each time period. For the comet assay, the microscope slides were precoated with 100 μl of 1% NMA in PBS, covered with a coverslip to obtain a uniform layer and then allowed to solidify overnight at 37 °C. For second layer preparation, 20 μl of whole blood or liver ($1–5 \times 10^4$ cells) homogenate was suspended in 100 μl of 0.5% LMA. The suspended cells and LMA were pipetted onto precoated slides and spread uniformly, and a coverslip was placed on them. The slides were then dried at 4 °C for 10 min. The third layer of plain 0.5% LMA (120 μl) was applied, and a coverslip was quickly put to get an even layer and dried at 4 °C. The coverslips were then removed, and 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 soaked for 20 min in alkaline buffer (10 M NaOH, 200 mM Na_2 EDTA, pH >13.0), and then, electrophoresis was carried out at 25 V adjusted at 300 mA for 20 min. The slides were neutralised twice in 0.4 M Tris buffer, pH 7.5, for 5 min and once in absolute methanol for 5 min. The slides were coded, stained with 20 μg/ml of ethidium bromide and screened using a fluorescence microscope (Olympus, Shinjuku-ku, Tokyo, Japan) with a blue (488 nm) excitation filter and a yellow (515 nm) emission (barrier) filter at ×400 magnifications. One hundred and fifty cells were randomly selected per rat (50 cells per slide) and used to quantify the DNA damage and expressed as a percentage of DNA in the comet tail. Measurement of DNA breakage was conducted 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 micronucleus test (MNT) was performed in bone marrow cells and peripheral blood (PB) cells of rats as per OECD guideline 474 (OECD 1997a, 1997b) with slight changes (Singh et al. 2016). The bone marrow cells were removed from thigh bones of the rats after acute oral exposure with WO_3 NPs and MPs for 24 and 48 h. The bone marrow was extracted by aspiration from both femur bones and put in the hypotonic solution consisting of 1% sodium citrate and centrifuged for 5 min at 1000 rpm. The pellet was resuspended in 1% sodium citrate, and a smear was prepared on a microscope slide and allowed to dry in humidified air overnight. The MNT in PB cells was conducted by following the procedure of Çelik et al. (2005). Whole blood was collected from retro-orbital plexus of each rat after administration at 48 and 72 h from each group. Smears were prepared on clean microscope slides and dried overnight. The slides were fixed in methanol for 2 min and stained for 3 min with 0.5% Giemsa stain, which was prepared in PBS. The stained slides were examined at ×1000 magnification for the presence of micronucleus (MN). Three slides were prepared for each animal. Two thousand polychromatic erythrocytes (PCEs) were analysed per

animal randomly from each of the three slides, and the frequencies of micronucleated PCEs (MN-PCEs) were documented. To determine the ratio of PCEs to normochromatic erythrocytes (NCEs) in the bone marrow and PB cells, 1000 cells from each animal were checked, and the ratio was expressed as the percentage: (PCEs × 100/PCEs + NCEs).

Chromosomal aberration assay

The protocol adopted by OECD guideline 475 (OECD 1997a, b) and Singh et al. (2013) was used for CA analysis. The assay was executed 18 and 24 h after oral treatment of female Wistar rats with 100, 500 and 1000 mg/kg bw doses of WO_3 NPs and MPs. Cell division was arrested with a 0.01 ml/g bw dose of a mitotic inhibitor colchicine (0.02%) administered IP, 2 h before sacrifice. For examination of cells in metaphase, the bone marrow was collected in a hypotonic solution of 0.9% sodium citrate from both femur and tibia bones and centrifuged at 2000 rpm for 20 min. Cells were then washed with a fixative consisting of ice-cold Carnoy's solution (methanol/acetic acid, 3:1 *v*/*v*) three times until the pellets were clean. The cells were refrigerated for 24 h and centrifuged and resuspended in fresh fixative. Two drops of the final cell suspension were dropped onto a chilled slide from a height of 30–40 cm. The slides were gently blown and dried on a hot plate at 40 °C. Three slides from each rat were readied and stained with 1% Giemsa for 2 h. Five hundred well-spread metaphases per dose (100 per animal) were chosen to identify the appearance of CAs. One thousand cells were used to determine the mitotic index (MI) for calculating the rate of cell division.

Biochemical assays

Whole blood was acquired from all rats by puncturing the retro-orbital plexus and separated batch wise. The samples were kept at room temperature for 45 min for the blood to clot. Serum was obtained from the blood without using anticoagulant by centrifuging at 2500 rpm for 15 min at room temperature and used for the estimation of AST and ALT. For determination of reduced GSH content, catalase and lipid peroxidation whole blood was used. After the blood samples were collected, the rats were sacrificed, and their livers were quickly removed and washed in ice-cold normal saline and then with 0.15 M Tris-HCl (pH 7.4), dried and weighed. Liver tissues from each group were immediately homogenised separately in cold 0.25 M of sucrose to make 10% homogenate (*w*/*v*) utilising a Miccra D-1 high-speed tissue homogeniser. The homogenate was then centrifuged at 10,000×*g* at 4 °C for 10 min to remove debris. The protein content in the serum and tissue supernatant was estimated using a standard protocol (Lowry et al. 1951). Activities of AST and ALT and measurement of GSH content, catalase and lipid peroxidation in serum and liver tissue of female Wistar rats treated with WO_3 NPs and MPs with 100, 500 and 1000 mg/kg bw at 24 and 72 h after treatment were determined using a spectrophotometer.

Aspartate aminotransferase and alanine aminotransferase activity

Hepatic markers were determined in serum and liver as per the protocol of Yatzidis (1960). Serum (0.05 ml) and 0.1 ml of liver tissue homogenate with 0.5 ml of substrate (AST and ALT in phosphate buffer pH 7.4) were incubated in water bath at 37 °C for an hour. Immediately after incubation, 0.5 ml of aniline citrate and 0.5 ml of 2,4-dinitrophenylhydrazine (DNPH) solution were added and mixed thoroughly to stop the reaction and kept for 5 min at room temperature. Next, 3 ml of 0.75 M NaOH was added to the mixture vigorously and incubated for 30 min and the absorbance was read at 500 nm in a UV-vis spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). AST and ALT activities were expressed as micromole per hour per millilitre in serum and micromole per hour per milligram protein in liver, respectively.

Reduced glutathione content

GSH content was estimated in liver tissue and blood utilising the procedure of Jollow et al. (1974). One gram of liver tissue from both treated and control group rats was rinsed in ice-cold physiological saline, perfused with cold KCl buffer (1.15% KCl and 0.5 mM EDTA) and homogenised in potassium phosphate buffer (0.1 M, pH 7.4, KPB) using a Miccra D-1 high-speed tissue homogeniser. An aliquot of 0.5 ml of tissue homogenate was then incubated with 0.5 ml of sulfosalicylic acid (4% *w*/*v*) for 1 h on ice and centrifuged at 10,000 rpm for 10 min. To the 0.4 ml aliquot of the supernatant, 0.4 ml of DTNB (4 mg/ml in 5% sodium citrate) was mixed, and then, 2.2 ml KPB (0.1 M, pH 7.4) was added. The yellow colour obtained was read at 412 nm. The quantity of GSH present was expressed as microgram GSH/g wet weight of tissue. For estimating the GSH content in blood, 0.2 ml of whole blood was added to 1.8 ml of distilled water and incubated for 10 min at 37 °C, which resulted in complete haemolysis. Three millilitres of 4% sulfosalicylic acid was then added and centrifuged at 2500 rpm for 15 min. Of the 10 mM solution of DTNB, 0.2 ml was added to the supernatant in the presence of phosphate buffer (0.1 M, pH 7.4). Absorbance was noted at 412 nm using the UV-vis spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). The GSH concentration was expressed as units per millilitre.

Catalase

Catalase activity in blood and liver tissue was determined at room temperature by adopting the procedure of Sinha (1972). 100 μl of 5% RBC haemolysate and 10% of liver tissue homogenate was incubated separately with 0.5 ml of H_2O_2 (0.2 M) at 37 °C accurately for 90 s with 0.01 M phosphate buffer (pH 7.4). The reaction was arrested by adding 5% dichromate solution. The samples were then incubated at 100 °C for 15 min in boiling water bath. The quantity of H_2O_2 consumed was measured by noting the absorbance at 570 nm using a UV-vis spectrophotometer (Molecular Devices, Sunnyvale, CA, USA). The enzyme activity was expressed as units per milligram protein.

Lipid peroxidation

Malondialdehyde (MDA), a lipid peroxidation end product in tissue homogenate, was measured in accordance with the protocol described by Wills (1969) with little modifications as per Ohkawa et al. (1979). A 200 μl aliquot of liver tissue homogenate was added to 2 ml of thiobarbituric acid (TBA), and trichloroacetic acid (TCA) reagent (0.375 and 15%, respectively) and its volume were made up to 3 ml with distilled water. The solution was then heated in a water bath at 95 °C for 20 min and cooled under tap water. The reaction product (TBA–MDA complex) was extracted by addition of 3 ml of *n*-butanol to the previous solution. The absorbance of the pink-coloured product in *n*-butanol was estimated at 532 nm using a spectrophotometer (SpectraMax Plus, Molecular Devices, USA). The quantity of MDA was determined using a molar extinction coefficient of 1.56×10^5 M/cm and expressed as nanomoles of MDA formed per gram wet weight of the tissue. Of 5% RBC haemolysate, 0.1 ml was added to 0.2 ml of 8.1% SDS (*w*/*v*) in the case of whole blood. The solution was incubated for 10 min, and 1.5 ml of 20% acetic acid (pH 3.5) was added. Next, 1.5 ml of 0.8% TBA (*w*/*v*) and 0.7 ml distilled water were added to the solution. Then, the mixture was incubated for 1 h in a boiling water bath. Later, 1 ml of distilled water was added to the solution after cooling and centrifuged at 6000 rpm for 15 min. The absorbance of the supernatant was recorded at 532 nm, and the values obtained were expressed as nanomoles of MDA per millilitre.

Tungsten content analysis in different tissues, blood, urine and faeces

The distribution pattern of the WO_3 NPs and MPs in the female Wistar rats was studied in brain, faeces, heart, kidney, liver, spleen, urine and whole blood. After treatment, the rats were placed in metabolic cages, and faeces and urine specimens were obtained at 6, 24, 48 and 72 h. The samples were processed as per the protocol of Gómez et al. (1997). Initially, the samples were digested in nitric acid for 12 h and then heated at 80 °C for 10 h and further heated at 130–150 °C for 30 min. A volume of 0.5 ml of 70% perchloric acid was then added to the samples. They were heated again at 130 °C for 4 h for drying. For analysis, the samples were filtered, and 2% of HNO_3 was added to make a final volume of 5 ml. The W standard solution was diluted (serially) to 1, 10, 50 and 100 ppm. The content of W in the samples was evaluated by ICP-OES (JY Ultima, Jobin Yvon, France) by the method of Sahoo et al. (2009).

Statistical analysis

All the experiments were performed in triplicates, and the results were expressed as the mean and standard deviation (mean ± SD) of the five animals ($n = 5$) for each group. For homogeneity of variance, data within each group was tested for outliers by means of the Grubbs test (Grubbs 1969). Mean differences between groups were determined by one-way analyses of variance (ANOVA) followed by Dunnett's multiple pair-wise comparison post-test analysis with confidence intervals of 95%. GraphPad Instat Prism 3 Software package for Windows (GraphPad Software, Inc., La Jolla, CA, USA) was used for analysing the data. Statistical significance was set at $P < 0.05$ ("*" represents $P < 0.05$ and "**" represents $P < 0.01$).

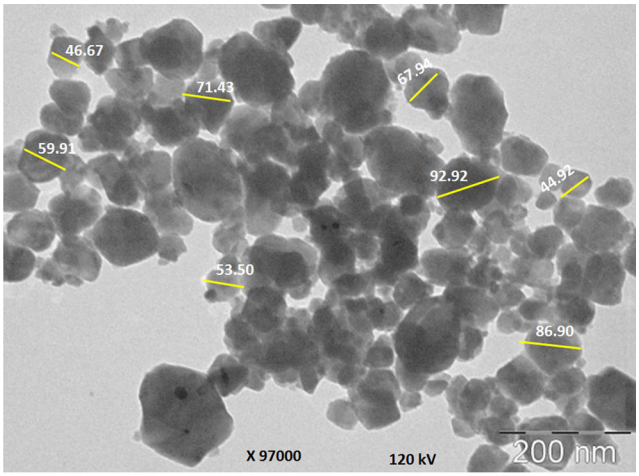
Results

Characterisation of tungsten oxide nanoparticles and microparticles

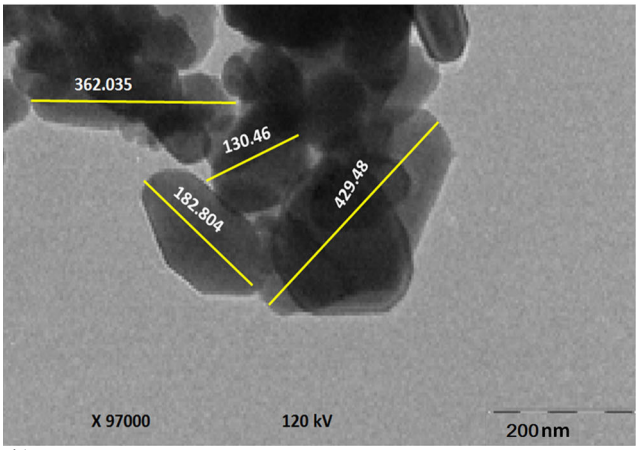
The size of WO_3 NPs and MPs was 53.2 ± 1.91 nm and 5.17 ± 3.18 μm, respectively, and showed spherical morphology (Fig. 1a, b). The hydrodynamic diameter and PdI of WO_3 NPs in Milli-Q water suspension obtained by DLS were 205.3 ± 1.6 nm and 0.407, respectively. The DLS data revealed larger values of the NP size in comparison to that obtained by TEM analysis, suggesting that WO_3 NPs produced larger agglomerates when suspended in water. The zeta potential (ζ) and electrophoretic mobility of WO_3 NPs were found to be −6.03 mV and −0.75 μm cm/s, respectively, at pH 7.0. For MPs, no data for DLS and LDV analyses could be obtained as it was out of the detection limits (Table 1).

Animal observation, food consumption, body weight, organ weight and histopathological evaluation

Female rats treated with single doses of 5, 50, 300 and 2000 mg/kg bw of WO_3 NPs and MPs showed no adverse symptoms or mortality after 14 days of oral exposure. Moreover, no significant changes were observed in food consumption, bw and relative organ weight (liver, kidney, lungs, heart, spleen and brain) in animals receiving acute doses of both the particles (data not shown). Thus, the acute oral toxicity of WO_3 NPs and MPs in rats was greater than 2000 mg/



a) WO_3 NPs



b) WO_3 MPs

Fig. 1 Characterisation of WO_3 NPs and WO_3 MPs in Milli-Q water mixing done via probe sonication for 10 min by TEM. **a** WO_3 NPs. **b** WO_3 MPs

kg bw, ranking WO_3 NPs and MPs in category 5 as per OECD guideline 420 and the Globally Harmonized System. The criteria for hazard category 5 are intended to enable the identification of test substances, which are of relatively low acute toxicity hazard but which, under certain circumstances, may present a danger to vulnerable populations. These substances are anticipated to have an oral LD_{50} in the range of 2000–5000 mg/kg bw. The slides of the organs from WO_3 NP- and MP-treated animals showed normal architecture of liver, kidney, spleen, heart and brain tissues. There was no significant difference between the control and WO_3 NP- and MP-treated rat tissues (Supplementary Fig. 1).

Genotoxicity analysis

Comet assay

Cell viability of PBL and liver cells of all the samples ranged from 88 to 97%, and there was no significant difference between PBL and liver cells of any of the samples when compared to respective controls (Table 2). A statistically significant increase in DNA damage (% tail DNA) was observed in the peripheral blood lymphocytes (PBL) and liver cells of female rats exposed to 1000 mg/kg bw dose with WO_3 NPs at the 24- and 48-h sampling times (Fig. 2a, b). An increasing trend in DNA damage was also found at the 6- and 72-h sampling times at 1000 mg/kg bw dose; however, the damage was not significant. An increasing trend in % tail DNA was also observed in animals treated with 100 and 500 mg/kg bw WO_3 NPs, but the increase was not statistically significant. An increasing trend in % tail DNA was also depicted in the PBL and liver cells after treatment with increasing doses of WO_3 MPs, but the increase was not statistically significant at all dose levels and time intervals. Further, the mean % tail DNA in CP-treated positive control group was increased significantly ($P < 0.01$) compared with the control.

Micronucleus test

After exposure of rats for 24 h to WO_3 NPs, bone marrow cells showed a statistically significant ($P < 0.05$) increase in MN-PCE frequency at the high dose of 1000 mg/kg bw only. However, no significant increases in MN-PCEs were observed at any of the other exposure times regardless of the dose tested. Rats treated with WO_3 MPs did not show any significant increase in the frequency of MN-PCEs at any of the doses and sampling periods in bone marrow cells (Table 3). On the other hand, the CP (40 mg/kg bw)-treated group had a significant ($P < 0.01$) increase in MN-PCE frequency. Similarly, significant ($P < 0.05$) increases in MN-PCE numbers after treatment with 1000 mg/kg bw of WO_3 NPs were observed after 48 h, but not in any of the other treatment groups, in PB cells (Table 4). The positive control rats treated

Table 1 Characterisation of WO_3 NPs and MPs

Particles	Size using TEM (nm)	DLS		LDV		
		Average diameter (nm)	PDI	Zeta potential ζ (mV)	Electrophoretic mobility (μm cm/V/s)	pH
WO_3 NPs	53.2 ± 1.91(nm)	205.3 ± 1.6	0.407	−6.03	−0.75	7.0
WO_3 MPs	5.17 ± 3.18(μm)	ND	ND	ND	ND	7.0

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

Table 2 Effect of various doses of WO_3-NPs and WO_3-MPs on cell viability

Treatments	Dose (mg/kg bw)	Cell viability (%)							
		PBL				Liver			
		6 h	24 h	48 h	72 h	6 h	24 h	48 h	72 h
Control[a]	Water	97.5 ± 1.52	97.9 ± 1.04	97.4 ± 1.4	97.6 ± 1.24	94.7 ± 0.58	94.2 ± 0.84	94.1 ± 0.18	94.4 ± 0.87
Wo_3 NPs	100	95.9 ± 1.6	95.5 ± 0.63	95.1 ± 0.3	96.1 ± 0.5	93.7 ± 1.4	91.9 ± 0.25	92.1 ± 0.36	92.8 ± 1.4
	500	95.5 ± 0.36	95.8 ± 0.32	96.1 ± 0.12	96.5 ± 0.41	92.1 ± 1.2	90.2 ± 0.6	91.7 ± 0.51	91.4 ± 1.7
	1000	93.2 ± 0.89	92.8 ± 0.2	92.9 ± 0.7	94.1 ± 0.9	90.4 ± 0.8	88.6 ± 0.4	90.9 ± 0.3	89.8 ± 0.9
Wo_3 MPs	100	96.1 ± 0.85	96.1 ± 0.23	96.7 ± 0.3	96.9 ± 0.63	94.3 ± 1.2	91.1 ± 1.4	91.9 ± 0.6	91.5 ± 0.4
	500	95.9 ± 0.74	95.7 ± 0.74	96.2 ± 0.74	96.1 ± 0.12	93.5 ± 0.6	91.5 ± 1.2	91.7 ± 0.7	91.8 ± 1.8
	1000	95.8 ± 0.1	94.9 ± 0.4	94.9 ± 0.4	95.6 ± 0.53	92.9 ± 0.52	90.74 ± 0.1	91.8 ± 0.63	92.3 ± 1.1

Data represented as mean ± SD. Cell viability was checked at each time interval by the trypan blue exclusion method

$n = 5$ animals per group

[a] Negative control (Milli-Q water)

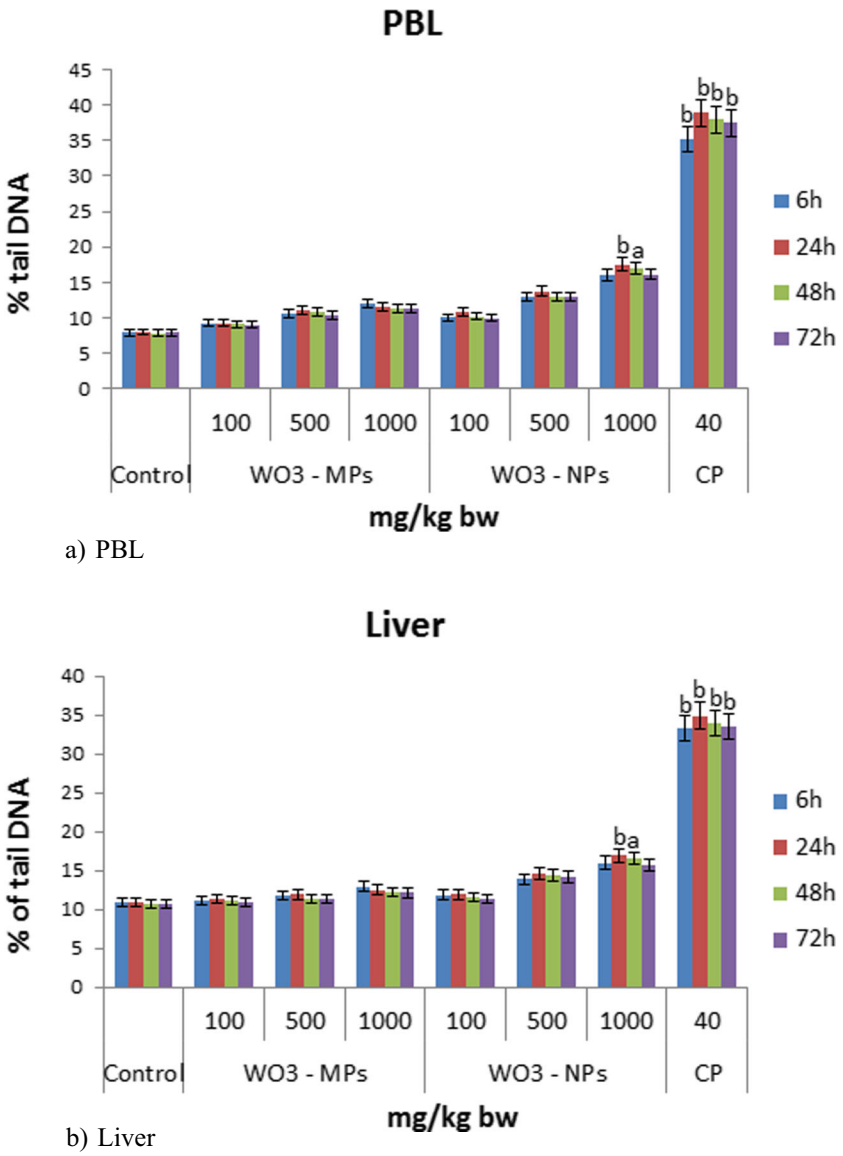


Fig. 2 The comet assay of WO_3-NPs and MPs. **a** Mean % tail DNA in PBL. **b** Mean % tail DNA in the liver at 6, 24, 48 and 72 h of female Wistar rats after single oral treatment. *Letters* indicate a significant difference ($^aP < 0.05$) and ($^bP < 0.01$) with respect to control group

Table 3 Frequency of MN-PCEs and % PCEs in bone marrow cells treated orally with the different doses of WO_3 NPs and MPs at 24 and 48 h in rats

Treatments	Dose (mg/kg bw/day)	Sampling times (h)			
		24		48	
		MN-PCEs	% PCEs	MN-PCEs	% PCEs
Control[a]	Milli-Q water	4.2 ± 1.7	40.1 ± 2.12	4.22 ± 0.1	40.17 ± 0.15
Wo_3 NPs	100	4.23 ± 0.2	39.97 ± 1.7	4.21 ± 1.17	39.71 ± 0.73
	500	4.90 ± 0.11	39.5 ± 0.62	4.72 ± 0.1	39.2 ± 1.9
	1000	5.38 ± 1.02*	38.3 ± 0.32	5.17 ± 1.24	38.7 ± 0.52
Wo_3 MPs	100	4.13 ± 0.17	40.0 ± 0.4	4.1 ± 1.72	40.1 ± 0.7
	500	4.25 ± 1.2	39.91 ± 0.2	4.21 ± 1.78	39.99 ± 0.54
	1000	4.51 ± 1.18	39.7 ± 0.6	4.34 ± 1.4	39.67 ± 1.3
CP[b]	40	30.2 ± 2.17**	25.21 ± 0.8**	27.7 ± 1.1**	24.12 ± 0.12**

Each value represented as mean ± SD of three biological replicates from each group, $n = 5$ animals per group

Asterisks indicate a significant difference (*$P < 0.05$) and (**$P < 0.01$) with respect to control group

[a] Milli-Q water (negative control)

[b] Cyclophosphamide (positive control)

with CP-induced had a more marked and significant increase in MN-PCEs and % PCEs. Exposure to 100 and 500 mg/kg bw of WO_3 NPs and to all doses of WO_3 MPs did not result in a significant decrease in % PCEs in PB cells in comparison to the negative control rats.

Chromosomal aberration assay

Exposure to WO_3 NPs induced a significant ($P < 0.01$) increase in CAs at 1000 mg/kg bw dose after 24 h, whereas at the 18-h sampling time, still a significant number of CAs were observed ($P < 0.05$) (Tables 5 and 6). A dose-dependent increase in numerical and structural CA was observed. However, WO_3 NPs did not induce a significant increase at 100 and 500 mg/kg bw at both sampling times. The CA assay conducted with rats exposed to WO_3 MPs showed structural (acentric fragment, breaks, gaps, minute and reciprocal translocation) and numerical (aneuploidy and polyploidy) alterations. However, significant increases in aberrant cells were not found regardless of dose or sampling time. The MI did not show any significant differences after exposure of rats to WO_3 NPs and MPs at 100, 500 and 1000 mg/kg bw compared with the control group.

Biochemical enzyme alterations

After exposure to 1000 mg/kg bw WO_3 NPs, there was a significant increase ($P < 0.01$) in AST and ALT levels in

Table 4 Frequency of MN-PCEs and % PCEs in rat's peripheral blood cells treated orally with different doses of WO_3 NPs and MPs at 48 and 72 h in female rats

Treatments	Dose (mg/kg bw/day)	Sampling times (h)			
		48		72	
		MN-PCEs	% PCEs	MN-PCEs	% PCEs
Control[a]	Milli-Q water	2.01 ± 0.1	3.97 ± 0.12	2.02 ± 0.11	4.07 ± 0.17
Wo_3 NPs	100	2.18 ± 1.22	3.75 ± 0.72	2.13 ± 1.27	3.94 ± 0.1
	500	2.32 ± 0.81	3.61 ± 1.23	2.22 ± 0.11	3.88 ± 0.23
	1000	3.08 ± 1.32*	3.17 ± 1.03	2.71 ± 1.02	3.51 ± 0.5
Wo_3 MPs	100	2.0 ± 0.1	3.91 ± 0.13	1.97 ± 0.14	3.90 ± 0.61
	500	2.14 ± 1.02	3.83 ± 0.2	2.11 ± 1.02	3.73 ± 0.42
	1000	2.82 ± 1.8	3.41 ± 0.14	2.54 ± 1.4	3.56 ± 0.7
CP[b]	40	10.2 ± 2.37**	1.9 ± 0.16**	9.5 ± 2.01**	2.26 ± 0.16**

Each value represented as mean ± SD of three biological replicates from each group, $n = 5$ animals per group.

Asterisks indicate a significant difference (*$P < 0.05$) and (**$P < 0.01$) with respect to control group

[a] Milli-Q water (negative control)

[b] Cyclophosphamide (positive control)

Table 5 Chromosome aberrations and percent MI were observed in bone marrow cells treated orally with different doses of WO_3 NPs and MPs at 18 h in rats

Dose (mg/kg bw/day)	MI (%), mean ± SD	Chromosomal aberrations							Aberrant cells (%)	Total cytogenetic changes	TA + gaps M ± SD	TA-gaps mean ± SD
		Numerical aberrations		Structural aberrations								
		Aneuploidy	Polyploidy	Gaps	Breaks	Minutes	Acentric fragments	Reciprocal translocations				
Control[a]	3.10 ± 1.2	0.4 ± 0.1	0.0 ± 0.0	0.48 ± 0.1	0.24 ± 0.2	0.3 ± 0.2	0.21 ± 0.0	0.0 ± 00	0.62 ± 0.7	2.1 ± 0.1	1.2 ± 0.1	1.0 ± 0.1
WO_3 NPs												
100	3.11 ± 0.19	1.0 ± 0.1	0.0 ± 0.0	0.70 ± 0.1	0.46 ± 0.1	0.4 ± 0.12	0.34 ± 0.21	0.0 ± 00	1.1 ± 0.1	2.6 ± 0.4	1.97 ± 0.8	1.6 ± 0.64
500	3.05 ± 0.2	1.91 ± 0.8	0.0 ± 0.0	1.51 ± 0.3	0.91 ± 0.4	0.51 ± 0.7	0.6 ± 0.4	0.0 ± 00	1.97 ± 0.9	3.68 ± 0.4	2.98 ± 0.1	2.08 ± 0.7
1000	2.97 ± 0.28	2.40 ± 1.0	0.0 ± 0.0	2.1 ± 0.5	1.6 ± 0.7	1.2 ± 0.6	1.7 ± 0.1	0.0 ± 00	2.1 ± 1.1*	4.0 ± 1.29*	3.4 ± 1.7*	2.6 ± 0.1*
WO_3 MPs												
100	3.10 ± 0.1	0.61 ± 0.4	0.0 ± 0.0	0.60 ± 0.1	0.39 ± 0.9	0.31 ± 0.1	0.31 ± 0.1	0.0 ± 00	1.01 ± 0.1	2.11 ± 0.1	1.51 ± 0.8	1.31 ± 0.4
500	3.01 ± 0.21	1.0 ± 0.18	0.0 ± 0.0	1.1 ± 0.4	0.74 ± 0.1	0.40 ± 0.1	0.59 ± 0.24	0.0 ± 00	1.50 ± 0.1	3.01 ± 0.7	1.75 ± 0.9	1.74 ± 0.4
1000	2.91 ± 0.21	1.90 ± 1.7	0.0 ± 0.0	1.89 ± 0.1	1.24 ± 0.4	1.0 ± 0.4	1.6 ± 0.61	0.0 ± 00	2.1 ± 0.7	4.2 ± 1.9	2.71 ± 1.4	2.4 ± 0.3
CP[b]	1.98 ± 0.1	34.1 ± 1.1**	2.3 ± 0.2**	10.1 ± 0.3**	7.7 ± 0.7**	9.6 ± 1.9**	10.1 ± 1.2**	1.2 ± 0.7**	30.1 ± 1.1**	88.2 ± 4.1**	41 ± 1.9**	31.4 ± 1.8**

From each rat, 100 metaphases were analysed, and each value represented as mean ± SD of three biological replicates from each group, $n = 5$ animals per group

TA total aberrations = structural aberrations

Asterisks indicate a significant difference (*$P < 0.05$) and (**$P < 0.01$) with respect to control group

[a] Negative control (Milli-Q water)

[b] Positive control (40 mg/kg bw)

Table 6 Chromosome aberrations and percent MI were observed in bone marrow cells treated orally with different doses of WO_3 NPs and MPs at 24 h in rats

Dose (mg/kg bw/day)	MI (%), mean ± SD	Chromosomal aberrations							Aberrant cells (%)	Total cytogenetic changes	TA + gaps M ± SD	TA-gaps mean ± SD
		Numerical aberrations		Structural aberrations								
		Aneuploidy	Polyploidy	Gaps	Breaks	Minutes	Acentric fragments	Reciprocal translocations				
Control[a]	3.10 ± 1.0	0.49 ± 0.1	0.0 ± 0.0	0.58 ± 0.1	0.29 ± 0.21	0.6 ± 0.19	0.23 ± 0.0	0.0 ± 00	0.69 ± 0.87	2.2 ± 0.4	1.29 ± 0.1	1.1 ± 0.1
WO_3 NPs												
100	3.01 ± 0.1	1.2 ± 0.7	0.0 ± 0.0	1.0 ± 0.1	0.69 ± 0.1	0.5 ± 0.72	0.37 ± 0.1	0.0 ± 00	1.1 ± 0.1	2.5 ± 0.42	2.01 ± 0.81	1.7 ± 0.69
500	2.97 ± 0.72	2.1 ± 0.7	0.0 ± 0.0	1.91 ± 0.7	1.5 ± 0.3	1.11 ± 0.4	0.7 ± 0.45	0.0 ± 00	2.3 ± 0.94	3.77 ± 0.40	3.08 ± 0.1	2.97 ± 0.77
1000	2.59 ± 0.21	2.6 ± 1.1	0.0 ± 0.0	2.3 ± 0.5	2.9 ± 0.17	2.2 ± 0.1	1.87 ± 0.17	0.0 ± 00	3.3 ± 1.7**	5.9 ± 1.2**	4.5 ± 1.1**	3.9 ± 0.3**
WO_3 MPs												
100	3.01 ± 0.17	1.0 ± 0.1	0.0 ± 0.0	0.62 ± 0.1	0.91 ± 0.4	0.42 ± 0.11	0.40 ± 0.71	0.0 ± 00	1.09 ± 0.1	2.11 ± 0.1	1.60 ± 0.02	1.73 ± 0.42
500	2.97 ± 0.7	1.42 ± 0.8	0.0 ± 0.0	1.23 ± 0.3	1.46 ± 0.1	1.1 ± 0.17	0.68 ± 0.1	0.0 ± 00	1.50 ± 0.14	3.07 ± 0.71	2.95 ± 0.41	2.86 ± 0.2
1000	2.79 ± 0.1	2.17 ± 1.7	0.0 ± 0.0	1.93 ± 0.3	2.7 ± 0.4	2.02 ± 0.44	1.69 ± 0.21	0.0 ± 00	2.10 ± 0.6	4.28 ± 1.1	3.91 ± 0.27	3.0 ± 0.7
CP[b]	1.98 ± 0.2	35.9 ± 1.7**	2.9 ± 0.27**	11.1 ± 0.1**	9.8 ± 0.7**	9.9 ± 1.7**	11.9 ± 1.3**	1.5 ± 0.1**	32.1 ± 1.31**	87.2 ± 2.7**	43 ± 1.5**	34.2 ± 1.2**

From each rat, 100 metaphases were analysed; each value represented as mean ± SD of three biological replicates from each group, $n = 5$ animals per group

TA total aberrations = structural aberrations

Asterisks indicate a significant difference (*$P < 0.05$) and (**$P < 0.01$) with respect to control group

[a] Negative control (Milli-Q water)

[b] Positive control (40 mg/kg bw)

serum and liver at the 24-h sampling time, whereas at 72 h, the increase was still significant but at a lesser P value ($P < 0.05$). Rats exposed to WO_3 NPs at 100 and 500 mg/kg bw and WO_3 MPs at all doses revealed a trend towards increased enzyme levels in a dose-dependent manner. However, these trends were not statistically significant in both serum and liver. After 24 h, the level of MDA was significantly increased ($P < 0.01$), and the levels of GSH and CAT were significantly decreased ($P < 0.01$) in serum. However, in the liver, the level of MDA was slightly increased, and the levels of GSH and CAT were reduced marginally, but the decrease was statistically still significant ($P < 0.05$) at the dose of 1000 mg/kg bw. At 72 h, GSH, CAT and MDA levels were still significant ($P < 0.05$) in the serum with WO_3 NPs (1000 mg/kg bw). However, in the liver, the levels of the three enzymes were raised showing an increasing trend. Further, the doses of 100 and 500 mg/kg bw of WO_3 NPs and all doses of WO_3 MPs did not reveal statistically significant increases in the level of MDA. Moreover, a decreasing trend was observed in the levels of GSH and CAT in serum and liver at both sampling times when compared to the untreated control group. However, the effect was not significant (Tables 7 and 8).

Tissue biodistribution of tungsten

W accumulated significantly ($P < 0.01$) in all tissues (liver, kidney, lung, spleen, heart) and whole blood of rats treated with WO_3 NPs at the dose of 1000 mg/kg bw at the 6-, 24- and 48-h sampling times (Fig. 3a–e). In contrast, at 72 h, the deposition was still significant ($P < 0.05$). At 6-, 24- and 48-h sampling times, there was significant accumulation in brain tissue, but not at 72 h (Fig. 3f). The greater level of W was reached at 24 h in all organs, followed by a decrease at later time points. The maximum amount of W was detected in the liver followed by blood, kidneys and lungs at 24 and 48 h (Fig. 3a, g, b, c). The distribution of W at all-time intervals was greater after treatment with 1000 mg/kg bw of WO_3 NPs followed by 500 and 100 mg/kg bw. In contrast, rats treated with WO_3 MPs did not exhibit significant W accumulation in any tissue and whole blood at any of the doses and time intervals. The excretion of W in urine was significant at all doses of WO_3 NPs at all sampling times in dose- and time-dependent manner, whereas 1000 mg/kg bw WO_3 MP-dosed animals showed still significance at all sampling times. There was no significant accumulation of W after treatment with 100 and 500 mg/kg bw of WO_3 MPs regardless of exposure time. The majority of W from both nanoparticle- and microparticle-treated animals was cleared through the faeces, and the clearance was reduced from 24 to 72 h (Fig. 3i).

Table 7 Biochemical and oxidative stress parameters in rats treated orally with different doses of WO_3 NPs and MPs at 24 h and 72 h in blood

Treatments	Dose (mg/kg bw)	AST (μmol/h/ml)		ALT (μmol/h/ml)		GSH (U/ml)		Catalase (U/mg protein)		MDA (nmol/ml)	
		24 h	72 h	24 h	72 h	24 h	72 h	24 h	72 h	24 h	72 h
Control	Milli-Q	2.10 ± 1.7	1.07 ± 1.1	1.5 ± 1.7	1.0 ± 1.0	3.91 ± 3.8	3.41 ± 1.0	111.1 ± 0.7	101.1 ± 0.71	2.13 ± 0.5	1.05 ± 1.5
WO_3 NPs	100	2.29 ± 0.1	1.21 ± 1.1	1.97 ± 1.0	1.17 ± 1.0	3.8 ± 1.2	2.88 ± 1.0	104.4 ± 2.1	94.14 ± 0.1	2.89 ± 0.6	1.75 ± 0.16
	500	2.57 ± 0.2	1.42 ± 0.2	2.27 ± 1.07	1.32 ± 0.7	3.14 ± 0.7	2.44 ± 0.1	93.4 ± 0.2	88.4 ± 0.21	3.45 ± 0.3	2.15 ± 0.3
	1000	3.13 ± 0.1**	1.93 ± 0.1*	2.77 ± 0.12**	1.49 ± 0.5*	2.91 ± 0.2**	2.10 ± 0.2*	85.7 ± 2.6**	71.2 ± 2.6*	5.93 ± 0.9**	3.97 ± 1.9*
WO_3 MPs	100	2.10 ± 0.14	1.03 ± 0.8	1.77 ± 1.4	1.02 ± 0.1	3.89 ± 1.1	2.98 ± 1.0	109.7 ± 0.1	98.11 ± 0.13	2.89 ± 0.6	1.45 ± 0.4
	500	2.41 ± 0.12	1.19 ± 0.8	2.20 ± 1.0	1.12 ± 0.2	3.48 ± 0.3	2.54 ± 0.1	99.1 ± 1.0	92.4 ± 0.23	3.45 ± 0.3	2.05 ± 0.2
	1000	3.01 ± 1.14	1.42 ± 0.81	2.40 ± 1.2	1.36 ± 0.19	3.31 ± 0.1	2.19 ± 0.2	93.1 ± 0.8	81.2 ± 2.16	3.93 ± 0.9	3.07 ± 0.3

Each value represented as mean ± SD of three biological replicates from each group, $n = 5$ animals per group

Asterisks indicate a significant difference (*$P < 0.05$) and (**$P < 0.01$) with respect to control group

Table 8 Biochemical and oxidative stress parameters in rats treated orally with different doses of WO_3 NPs and MPs at 24 h and 72 h in the liver

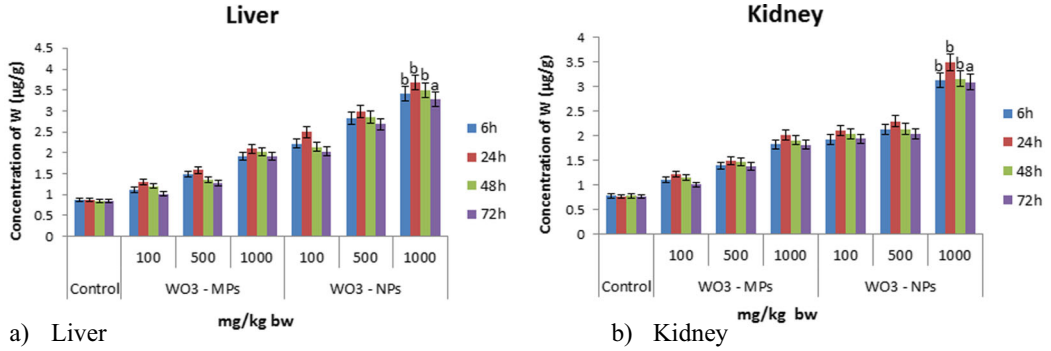
Treatments	Dose (mg/kg bw)	AST (μmol/h/mg protein)		ALT (μmol/h/mg protein)		GSH (μg/g tissue)		Catalase (U/mg protein)		MDA (nmol/g)	
		24 h	72 h	24 h	72 h	24 h	72 h	24 h	72 h	24 h	72 h
Control	Milli-Q	0.28 ± 0.11	0.29 ± 0.01	0.25 ± 0.17	0.25 ± 1.12	0.57 ± 0.8	0.56 ± 0.01	100.2 ± 1.3	100.5 ± 0.1	1.83 ± 0.15	1.85 ± 0.2
WO_3 NPs	100	0.30 ± 0.71	0.29 ± 1.11	0.27 ± 0.01	0.25 ± 1.21	0.55 ± 1.21	0.55 ± 0.21	94.1 ± 0.1	94.14 ± 0.01	1.97 ± 0.2	1.95 ± 0.06
	500	0.37 ± 0.32	0.32 ± 0.3	0.29 ± 1.01	0.27 ± 0.02	0.51 ± 0.7	0.54 ± 0.11	89.1 ± 0.23	88.4 ± 1.13	2.64 ± 0.2	2.15 ± 0.03
	1000	0.42 ± 0.1**	0.38 ± 0.2*	0.31 ± 0.3**	0.29 ± 0.2*	0.47 ± 0.1*	0.50 ± 0.3	87.1 ± 1.1*	81.2 ± 1.7	3.49 ± 0.95*	3.47 ± 1.01
WO_3 MPs	100	0.30 ± 0.04	0.28 ± 0.2	0.26 ± 1.04	0.25 ± 0.13	0.57 ± 1.01	0.56 ± 0.2	91.2 ± 0.1	88.11 ± 1.03	1.77 ± 0.16	1.31 ± 0.10
	500	0.31 ± 0.14	0.30 ± 0.3	0.27 ± 1.02	0.26 ± 0.7	0.53 ± 0.02	0.54 ± 0.3	84.01 ± 1.02	82.4 ± 1.23	2.21 ± 0.1	2.01 ± 0.02
	1000	0.34 ± 1.04	0.32 ± 0.2	0.28 ± 1.9	0.26 ± 0.03	0.51 ± 0.31	0.50 ± 1.12	80.3 ± 0.3	67.2 ± 1.19	3.19 ± 0.01	2.77 ± 0.13

Asterisks indicate a significant difference (*$P < 0.05$) and (**$P < 0.01$) with respect to control group, each value represented as mean ± SD of three biological replicates from each group, $n = 5$ animals per group

Discussion

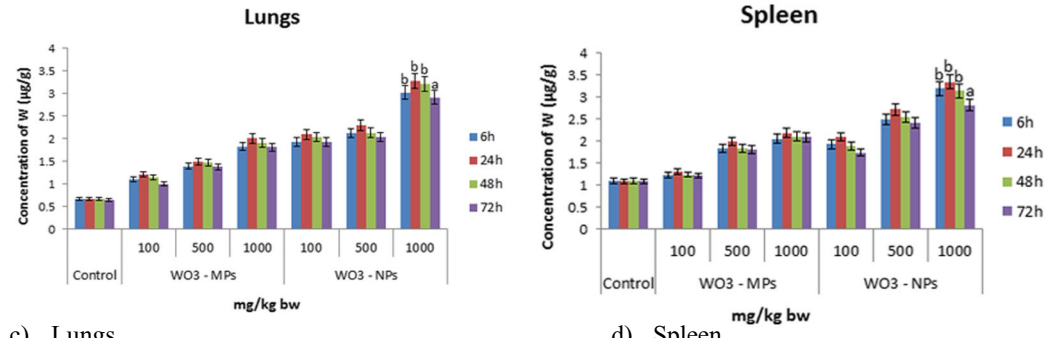
Nanotechnology is likely to have a vital role in the world's economy and also in our day-to-day lives. In recent years, the application of WO_3 NPs has acquired significance in the industrial, military and aerospace sectors due to their surface-to-volume ratio and high thermal stability (Zhou et al. 2012). However, the possible harmful effects of WO_3 NPs have not been investigated thoroughly. Therefore, our study focused on assessing the acute oral toxicity of WO_3 NPs and MPs in female rats. Currently, there are no guidelines for in vivo toxicity assessment of NPs. Hence, we chose to work with the doses (5, 50, 300 and 2000 mg/kg bw) suggested by OECD guideline 420 (OECD 2001). Further, the doses at which humans may be exposed to WO_3 NPs in the environment are yet to be established. The single oral treatment with various doses of WO_3 NPs and MPs in female Wistar rats did not result in an apparent adverse outcome. Moreover, histopathological studies of WO_3 NP- and MP-treated rats did not show any obvious adverse effects. The experimental outcome gives preliminary information on the genotoxicity of WO_3 NPs.

Genotoxicity studies provide an estimation of different types of genetic damage after exposure to test substances. They are important for risk assessment of potential carcinogens. NPs can penetrate into cells and bind to the macromolecules such as DNA and protein (AshaRani et al. 2008). Several types of NPs have been known to induce genotoxic effects (Klien and Godnić-Cvar 2012; Magdolenova et al. 2014). Therefore, genotoxicity assays were used to further our understanding of the genotoxic potential of NPs. In the present study, we demonstrated that 53 nm WO_3 NPs induced significant DNA damage via comet assay in rats exposed to 1000 mg/kg bw dose at 24 and 48 h post-treatment in PBL and liver cells in comparison to controls. There was a diminution in the DNA damage with time, which may be related to an increase in the activity of the DNA repair process of the animals after the extended exposure time (Karlsson 2010). However, oral administration of WO_3 NPs at 500 and 100 mg/kg bw doses and WO_3 MPs at all three doses did not reveal a significant increase in % tail DNA at all sampling times. In vivo genotoxicity studies with WO_3 NPs using the comet assay by oral route have not been reported. However, in an in vitro study, cultured human lymphocytes treated with 200, 400 and 500 μM of WO_3 NPs caused a significant increase in DNA damage (Akbaba et al. 2016). The MNT results showed the formation of MN in MN-PCEs and PB cells in a time-dependent and dose-dependent manner after treatment with WO_3 NPs and MPs. A significant increase in MN frequency was observed at 24 h after treatment with 1000 mg/kg bw of WO_3 NPs in comparison to control. Likewise, in a different study, exposure to doses of 50 and 100 mg/kg bw caused a significant increase in MN frequency in Sprague-Dawley rats treated with WO_3 NPs daily by IP route for



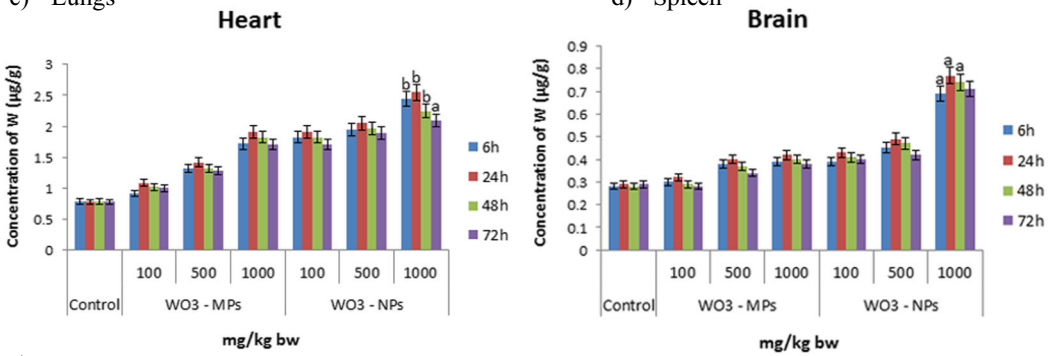
a) Liver

b) Kidney



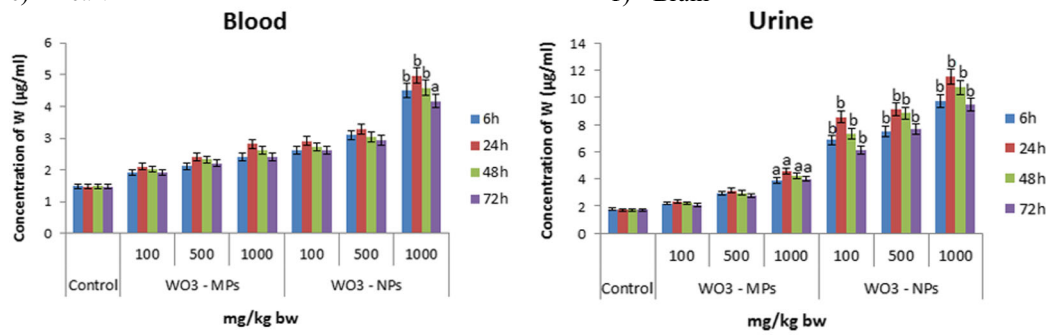
c) Lungs

d) Spleen



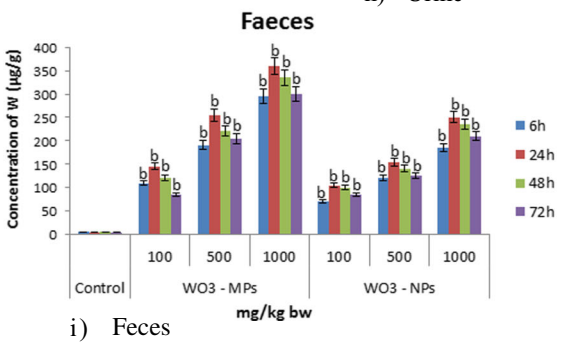
e) Heart

f) Brain



g) Blood

h) Urine



i) Feces

◀ **Fig. 3** Tungsten distribution was measured by ICP-OES in **a** liver, **b** kidneys, **c** lungs, **d** spleen **e** heart, **f** brain, **g** blood, **h** urine and **i** faeces of rats with 100, 500 and 1000 mg/kg bw of WO_3-NPs and MPs after 6, 24, 48 and 72 h of single oral administration. *Letters* indicate a significant difference ($^aP < 0.05$) and ($^bP < 0.01$) with respect to control group

30 days (Turkez et al. 2013). Bone marrow CA analysis after exposure to WO_3 NPs indicated that these NPs induced a significant increase in CAs at the 1000 mg/kg bw dose at 24 h after exposure. However, MPs did not produce any significant effect in all the doses and sampling times. On the contrary, no increase in the incidence of CA was observed after IP injection with 25, 50 and 100 mg/kg bw doses of WO_3 NPs for 30 days (Turkez et al. 2013). Studies using different NPs showing size-dependent genotoxicity are available. MnO_2 45 nm significantly increased DNA damage in PBL and MN and increased CAs in the bone marrow cells at 1000 mg/kg bw (Singh et al. 2013). Similarly, 1000 mg/kg bw of CeO_2 NPs produced significant DNA damage in PBL and liver cells, MN formation in bone marrow and blood cells and cytogenetic changes in bone marrow (Kumari et al. 2014).

Analysis of AST and ALT is a standard method to measure hepatotoxicity. In the current study, treatment of rats with WO_3 NPs and MPs resulted in an increased enzymatic activity in serum and liver. However, significant increases were observed only at 24 h after treatment with 1000 mg/kg bw of WO_3 NPs. Similar increased enzyme levels of AST and ALT in liver and serum were found in Fe_2O_3 NP-treated rats after an acute oral exposure (Kumari et al. 2013). Chen et al. (2009) demonstrated significantly increased levels of serum AST and ALT after treatment of mice with TiO_2 NPs. Liu et al. (2011) and Wang et al. (2006) showed increases in levels of serum ALT in mice treated with TiO_2 and ZnO_2 NPs, respectively. At 72 h, the AST and ALT levels were decreased in a time-dependent manner. In concurrence with our results, a study showed that when monodispersed iron oxide (IO) formulations (10 mg/ml) were injected into mice by IV route, increased levels of AST and ALT at 6 to 24 h were observed. The enzyme levels returned to normal within 3 day post-injection. For all IO NP formulations, the total mass of iron accumulated in the liver decreased with time (Jain et al. 2008). The results of the current study suggested that the increase in the activity of AST and ALT at 24 h may be connected to the cellular toxicity of the liver of the treated animals (Adeyemi and Akanji 2011). However, the AST and ALT levels showed a decreasing trend at 72 h, indicating that the transient increase in the liver enzyme levels was not enough to damage the liver. This was also evident from the lack of significant changes in the histopathology of the liver and other tissues after single oral treatment with WO_3 NPs and MPs.

Oxidative stress is one of the mechanisms suggested for carcinogenicity (De Berardis et al. 2010). In the current investigation, MDA, an oxidative stress indicator was assessed for the induction of NP-mediated oxidative damage in rats. The endogenous antioxidant system comprising GSH and CAT plays an important role in free radical and peroxide metabolism and is responsible for protecting the cells against oxidative stress (Wang et al. 2006). A significant increase in MDA levels was observed in the serum and liver at 1000 mg/kg bw dose of WO_3 NPs, indicating induction of free radical generation. Correspondingly, the GSH and CAT levels were reduced after WO_3 treatment in a dose- and time-dependent manner. This may be probably due to the increased use of these enzymes in neutralising the free radicals generated. It has been demonstrated that interaction of NPs with enzymes and proteins causes alterations in the oxidative defence system leading to reactive oxygen species (ROS) generation (Schrand et al. 2010). When excessive ROS is generated; the levels of MDA may be enhanced, and the GSH level may reduce, indicating that the WO_3-treated animals suffered oxidative stress situation. The present data implies that it is likely that the antioxidant system of the rats was overwhelmed, resulting in a collapse of the antioxidant response and an increase in oxidative stress. Similar studies demonstrating oxidative stress induced with WO_3 NPs after acute oral treatment in rats have not been found in the literature. However, studies of WO_3 NPs in cultured rat liver cells showed dose-dependent negative alterations in oxidative status and antioxidant capacity levels (Turkez et al. 2013). When mice were treated with IO by IV route, the liver showed a marginal increase in oxidative stress despite a greater localisation of iron compared with other tissues. This observation suggests that the oxidative response depends on the capacity of individual tissues to neutralise the effect of stress conditions caused by the NPs. Given its main role in detoxification, the liver is exposed to many sources of oxidative stress and may have a greater capacity to neutralise these effects. It appears that nonmagnetic NPs also induce oxidative stress (Jain et al. 2008). Fe_3O_4 NP exposure following acute inhalation markedly increased MDA concentration whereas GSH and CAT activity showed a significant decrease after 24 h (Srinivas et al. 2012). Significant inhibition in GSH content in the liver at 1000 mg/kg bw oral dose of CeO_2 NPs was observed after 24 h whereas still significant depletion after 72 h in comparison to control was reported (Kumari et al. 2014). Further, a study has reported that the uptake of polymeric NPs (poly(isobutyl cyanoacrylate)) by Küpffer cells in the liver following IV administration induced modifications in the hepatocyte antioxidant system, such as depletion of GSH and catalase and peroxidation of lipids, but the events were reversible (Fernández-Urrusuno et al. 1997). The results of this study support our findings.

NPs have various types of absorption, biopersistence, distribution and elimination when compared to MPs because of their strong interaction with animal systems (Oberdörster et al. 2005). Therefore, to obtain a perception of the uptake, retention and elimination of NPs, biodistribution investigations were also conducted. The tissue distribution and localisation

of W were evaluated using ICP-OES after 6, 24, 48 and 72 h after acute oral administration with WO_3 NPs. The W concentrations were highly significant in various tissues and slightly significant in the brain. The maximum amount of W was found in the liver followed by blood, kidneys, lungs, spleen, heart and brain. The accumulation was dose and time dependent. Our results indicated that WO_3 NPs could effortlessly negotiate the gastrointestinal barrier and retain in the various organs. The excretion pattern showed that a little quantity of W from WO_3 NP-dosed animals was removed through the urine whereas a substantial amount of W was excreted via faeces from 6 to 72 h, indicating that as the days passed, the concentration of W was reduced and was removed by the excretion system of rats. W content analysis in rats orally dosed WO_3 NPs has not been done previously. However, our result is similar to earlier studies in which Fe_2O_3, MnO_2, CeO_2, TiO_2, ZnO and Cu NPs orally exposed to mice and rats accumulated in various tissues (Wang et al. 2007; Li et al. 2012; Singh et al. 2013; Chen et al. 2006; Kumari et al. 2014). The distribution of W after WO_3 MP treatment was compared between experiment groups and controls. The accumulation was not statistically significant in all the tissues at all three doses and time periods. Due to their larger size, WO_3 MP-treated rats probably showed more excretion in faeces at all doses and sampling periods.

The generation of ROS and induction of oxidative stress may be the major factors contributing to the toxicological mechanisms of NPs. The superoxide anion radicals, hydroxyl radicals and hydrogen peroxide are the important components of ROS (Sarkar et al. 2014). NPs can easily penetrate the cell membrane and can evoke a variety of biological stress reactions including oxidative stress (AshaRani et al. 2008). Slight genotoxicity found in the current study with WO_3 NPs could be due to the ability of NPs to attack the 5′ phosphate group of DNA to catalyse a break or through inflammation and ROS generation (Li et al. 2008; Chen et al. 2006). However, the probable mechanism for genotoxicity after different-sized particle exposure could also be oxidative stress-related DNA damage (Oberdörster et al. 2005). From our results, it can be assumed that an adverse effect of WO_3 NPs was via its ions and was related to its biodistribution and accumulation patterns. Further, the toxicity associated with nano-WO_3 may be linked to its ability to enter the rat cells (Shang et al. 2014). The alterations in biochemical indices such as reduction in liver and serum GSH content and increase MDA levels can be most probably due to the accumulation of W in these tissues.

Conclusion

The data from the current investigation suggested a comparatively low toxicity risk in rats with nano- and micro-WO_3, which is in accordance with the tested doses of other NPs (CeO_2, MnO_2 and Fe_2O_3) after single oral exposure. Therefore, WO_3 NPs and MPs can be considered in category 5 as per Globally Harmonized System for chemical substances. The results obtained in the present study indicated that WO_3 NPs induced genotoxicity as well as biochemical changes only at the higher dose of 1000 mg/kg bw. Furthermore, the NPs showed more accumulation when compared with MPs. It is apparent from this study that WO_3 NPs induce different results in comparison to WO_3 MPs. The observed toxicity may be due to oxidative stress and inflammation though the exact mechanism is not fully understood. The concentrations (100, 500 and 1000 mg/kg bw) were selected to see the effect on the likelihood of exposure to the humans when large quantities of NPs are released accidentally or intentionally into the environment. The tested doses do not necessarily reflect the actual concentrations of WO_3 NPs found in the real environment. Nevertheless, they can be used to assess the potential health risks associated with exposure to WO_3 NPs. However, more investigations are needed for careful assessment to ensure the safety of WO_3 NPs during production and applications. Hence, further repeated dose and chronic studies are warranted.

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Compliance with ethical standards All procedures of the study were reviewed and approved by the Institutional Animal Ethics Committee (IICT//TOX/PG/25/06/2014/01).

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Conflict of interest The authors declare that they have no conflict of interest.

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