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Toxicological assessment of nano and micron-sized tungsten oxide after 28 days repeated oral administration to Wistar rats

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ABSTRACT

Tungsten oxide (WO_3) nanoparticles (NPs) are being used in various applications. However, the health consequences of WO_3 NPs exposure have not been explored extensively. Hence, the goal of this study was to evaluate the toxicity of WO_3 NPs and their microparticles (MPs) after 28 days repeated oral administration in Wistar rats. The particles were characterised by transmission electron microscopy (TEM), dynamic light scattering (DLS), laser Doppler velocimetry (LDV), Brunner-Emmett-Teller (BET), X- ray diffraction (XRD), and inductively coupled plasma optical emission spectrometer (ICP-OES). Genotoxicity was determined using comet assay in blood and liver and micronucleus test in bone marrow. Biochemical parameters such as aspartate aminotransferase and alanine aminotransferase in serum and reduced glutathione content, catalase and lipid peroxidation in liver tissue were determined. Histopathological changes in tissues were documented. Biodistribution of tungsten (W) in rat's blood, urine, feces and tissues were analysed. The mean size of WO_3 NPs and MPs by TEM was 52 ± 2.97 nm, and 5.73 ± 7.58 µm and morphology were spherical in both the particles. DLS of NPs was 195.6 nm. XRD and BET data of WO_3 NPs and MPs showed a hexagonal and tetragonal crystal structure and surface area of 19.33 and 15.15 (m^2/g), respectively. The results revealed a significant increase in DNA damage and micronuclei, a difference in biochemical levels and histopathological alterations after exposure to 1000 mg/kg dose of WO_3 NPs. W biodistribution was detected in all the tissues in a dose and organ-dependent manner in both the particles. The highest amount of W was found in the liver and lowest in the brain of the treated rats. The tested NPs were found to have little toxicity hazard.

1. Introduction

Significant development and impressive profit have been achieved in the field of nanotechnology. One angle of this commercial enterprise consists of manufacturing nanoparticles (NPs). The properties exhibited by NPs are different from their microparticles (MPs) (> 100 nm) because of their size, structure and high surface area [1]. During the last couple of years, more than 1800 NP containing goods have become available in the market [2,3]. Hence, NPs may gain access to the human body through dermal, inhalation and oral routes during manufacture, use and disposal [4]. Since the NPs are found to be stable, it is anticipated that they may be retained in the human tissues and the environment for a long time. The likely toxicological effects of exposure to NPs are not well explored [5,6]. However, the limited literature indicates potential toxic side effects [7]. Acute and chronic toxicity to humans and environment induced by NPs have recently become a serious concern. Several investigations have evaluated the *in vivo* toxicity of NPs. The results revealed significant genotoxicity and biochemical alterations [8–11]. Further, a few studies have shown that NPs cause oxidative stress [12–15]. The actual mechanism of the carcinogenic effect of metals has not yet been studied. However, there is sufficient evidence to suggest that reactive oxygen species (ROS) play an important role [16]. Thus there is an urgent need to develop rapid, accurate and efficient testing strategies to assess toxic effects of these NPs.

Among several metal oxide NPs, tungsten oxide (WO_3) NPs have attracted an increasing interest due to their large surfaces and high-temperature stability in nanotechnological products [17]. They have

Abbreviations: WO_3 NPs, tungsten oxide nanoparticles; WO_3 MPs, tungsten oxide microparticles; TEM, transmission electron microscopy; DLS, dynamic light scattering; LDV, laser doppler velocimetry; XRD, X-ray diffraction; BET, Brunner–Emmett–Teller technique; ICP-OES, inductively coupled plasma optical emission spectrometer; OECD, Organization for Economic Co-operation and Development; MNT, micronucleus test; % PCE, percentage of polychromatic erythrocytes; MN-PCE, micronucleated polychromatic erythrocytes; MI, mitotic index; bw, body weight; RBC, red blood cells; HGB, hemoglobin; HCT, hematocrit; PLT, platelets; WBC, white blood cells; ANOVA, analysis of variance

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well-known electrical, gasochromic, mechanical, and photo-electrochromic properties that can be used in various applications. WO_3 NPs are very useful as field emission devices, optical devices, light – emitting diodes, chemical sensors, and gas sensors for the determination of ammonia, hydrogen and nitrogen [18–22]. WO_3 NPs have gained considerable importance for discoloration of rhodamine B dye [23]. Further, they have been identified in strategic sectors like aerospace for various engine components, nose cones, jet vanes and kinetic energy projectiles [24]. Besides, WO_3 NPs inhibited the bacterial growth in patients having urinary tract infection with indwelling catheters and gave continuous protection to the site [25].

Possible health impact of WO_3 NPs on humanity and the environment is of great concern due to their increased use. Although nanoscale WO_3 are being used commercially, few studies have demonstrated that exposure to WO_3 NPs may lead to adverse effects. In an *in vitro* study, WO_3 NPs showed a positive mutagenic response in *Salmonella typhimurium* bacterial strains (TA1537 and TA98) using Ames test [26]. Cultured rat primary hepatocytes were used to evaluate the cytotoxicity and DNA oxidation of WO_3 NPs. There was a significant decrease in cell viability and an increase in 8- oxo-2-deoxy guanosine levels [27]. Eleven metal oxide NPs along with WO_3 NPs were examined for cell viability in A549, Caco-2 and 3T3 cell lines. The WO_3 NPs showed a toxic effect (> 100 μg/ml) with neutral red uptake assay [28]. The genotoxic potential of WO_3 NPs was evaluated in bone marrow cells of Sprague-Dawley rats by intraperitoneal (IP) injection daily for 30 days. Significant increase in MN was noticed [29]. Prajapati et al. [30] investigated the pulmonary toxicity of WO_3 NPs in golden syrian hamsters after inhalation for 4 h/day for 4 and 8 days of exposure. The NPs caused significant cytotoxicity, morphological changes in the lung tissue at both the exposure periods. *In vivo* toxicology of WO_3 NPs by oral route has not been reported till date. In view of the above fact, the current study was undertaken to investigate the 28 days repeated oral toxicity study of WO_3 NPs and MPs in albino Wistar male and female rats. The particles were initially characterised by using dynamic light scattering (DLS), laser doppler velocimetry (LDV), transmission electron microscopy (TEM), X- ray diffraction (XRD), Brunauer–Emmett–Teller (BET) and inductively coupled plasma optical emission spectrometer (ICP-OES) before conducting the main study. Genotoxicity was investigated using comet and micronucleus tests. Haematological limits like red blood cells (RBC), white blood cells (WBC), haemoglobin (Hb), haematocrit (HCT) and mean corpuscular volume (MCV) were estimated. Biochemical parameters such as aspartate aminotransferase (AST) and alanine aminotransferase (ALT) reduced glutathione (GSH) content, catalase and lipid peroxidation were determined. Histopathological changes were examined in rat tissues. Furthermore, biodistribution of tungsten (W) in rat's whole blood, urine, feces and tissues were analysed using ICP-OES after 28 days of treatment.

2. Materials and methods

2.1. Chemicals

All the chemicals were purchased from Sigma Chemical Co. Ltd (St Louis, MO, USA). However, Perchloric acid, Triton X-100, DMSO was obtained from Hi-media Laboratories (India) and were of analytical grade. Glassware and Plasticware were purchased locally.

2.2. NPs and MPs

WO_3 NPs ($WO_3 < 100$ nm, 99.8% purity, CAT No. 550086) and WO_3 MPs ($WO_3 < 20$ μm, 99% purity, CAT No. 232785) (according to the manufacture's datasheet) were purchased from Sigma Chemical Co. Ltd (USA).

2.2.1. Characterization of particles

WO_3 NPs and MPs were characterised using BET, DLS, ICP-OES, LDV, XRD and TEM to evaluate the specific surface area, size distribution and zeta potential, purity analysis, state of dispersion, crystal structure and material size.

2.2.2. TEM of WO_3 particles

A TEM was utilised to attain the size and morphology of WO_3 NPs and MPs. The images obtained from the TEM (JEOL, JEM-2100, Japan) at an accelerating voltage of 120 kV. The microscope has a plunge freezer and a cryo transfer holder to fix samples in frozen condition and fitted with a Gatan 2 K × 2 K CCD camera for acquiring high-resolution images. The WO_3 particles were suspended in water (0.01 mg/ml), and one drop of suspension was placed on a carbon-coated copper TEM grid and evaporated at room temperature. The NPs were examined by using advanced microscopy techniques software for the digital TEM camera. This software was calibrated for nanoscale size measurements. For the size determination, 100 particles were calculated from random fields of view and images showing the general morphology of the particles.

2.2.3. DLS and LDV of WO_3 particles in solution

The size of the particles and agglomerates in solution was determined by DLS and LDV with a Zetasizer Nano-ZS from Malvern Instruments (Malvern Instruments, UK). The device has a 4 mW He-Ne 633 nm laser and an electric field generator to measure the samples for LDV. Freshly prepared WO_3 particle suspensions (20 μg/ml in Milli-Q water) were ultrasonicated for 10 min at 90% amplitude, 100 W and 30 kHz using a probe sonicator (UPH 100, Germany). The samples were then transferred to a 1.5 ml cuvette for DLS measurement thereupon 1 ml was transferred to a Zeta Potential cell for LDV. The instrument software was used to evaluate the mean particle diameter from the distribution of particles by the polydispersity index (PdI) which was a criterion for determination of the size ranges of the particles present in the solution. The PdI scale ranged from 0 to 1 where 0 indicates monodispersed and 1 indicates polydispersed state of particles.

2.2.4. BET analysis

The specific surface area (m^2/g) of the particles was determined by N_2 adsorption– desorption measured at 77 K according to the BET protocol using a Quadrusorb-SI V 5.06 analyser (M/S Quanta chrome Instruments Corporation, USA).

2.2.5. XRD of WO_3 particles

The XRD pattern of the WO_3 particles was documented on a Bruker AXS D-8 Advance powder X-ray diffractometer (Shimadzu, Japan). The instrument was operated at a current of 30 mA, voltage of 40 kV and utilizing a CuKα radiation ($\lambda = 1.5406$ Å). The diffractometer was controlled with Datascan software and the scan specifications were set at a scan rate of 1.2° per minute and scan range was $2\theta = 0–80°$.

2.2.6. Purity analysis

The ICP-OES analysis was performed for the purity of the WO_3 NPs according to the methodology described by Yokel et al., 2009. The suspensions (10 mg/10 ml in Milli-Q water) of the particles were ultrasonicated for 10 min with a probe sonicator (UP100H, Hielscher Ultrasonics GmbH, Teltow, Germany) at 90% amplitude (100 W, frequency 30 kHz). The particles were then analysed by ICP-OES.

2.3. Animals

Female and male albino Wistar rats were procured from the National Institute of Nutrition, Hyderabad, India. The rats obtained were 6–8 weeks old and weighed 100–120 g. The animals were acclimatised in our animal house facility in polypropylene cages (5 per group). The animals had access to unlimited standard laboratory pellets for food and tap water for drinking. They were maintained under standard conditions of automated light cycles (12 h light/12 h dark), humidity (55–65%) and temperature (22 ± 3 °C). The study protocols

were approved by the Institutional Animal Ethics Committee (IICT/BI0/TOX/PG/8/02/2013).

2.4. Treatment of animals

One set of animals (male and female) was used in the study (n = 80). Animals were randomly divided into 5 groups (10 rats; 5 males and 5 females in each group), one control group, three experimental groups (for each particle) consisting of low, medium and high doses of 250, 500 and 1000 mg/kg bw/day and one positive control group. Prior to treatment, rats were weighed and the volume of WO_3 suspension was calculated for each rat. Fresh WO_3 NP and MP particle suspensions were prepared daily in Milli-Q water, ultrasonicated for 10 min at 90% amplitude, 100 W and 30 kHz using a probe sonicator (UPH 100, Germany) and vortexed for proper mixing and they were administered orally every day for 28 days using a suitable intubation cannula with dosing volume 2 ml/100 g/bw. Each control rat received the same volume of Milli-Q water daily. The animals were kept in individual metabolic cages during the course of treatment to collect the urine and feces specimens. The dose selection was on the basis of an initial range-finding study, i.e acute toxicity study (data not shown). The highest dose was selected based on induction of a toxic effect without severe suffering or mortality, whereas, the lowest dose did not reveal any adverse effects. For genotoxicity studies, a single dose of 40 mg/kg bw of cyclophosphamide (CP) was used as a positive control and a volume of 0.01 ml/g bw was injected intraperitoneally 24 h before sacrifice. Feed consumption and bw was monitored weekly for four weeks. Twenty-four hours after the last dose, the treated rats were sacrificed by cervical dislocation.

2.5. Genotoxicity

2.5.1. Comet assay

For the evaluation of DNA damage, the alkaline comet assay was conducted at 24 h after repeated oral exposure with WO_3 NPs and MPs. The 24 h time point to analyse the liver tissue for comet assay was based on a pilot study where samples were analysed at 4, 24, 48, & 72 h to identify an optimal time for DNA damage induced by WO_3 NPs and MPs (data not shown). Hence, in the current study, whole blood and liver tissues were collected at 24 after the last dose. Whole blood was withdrawn from retro-orbital plexus (in heparinised tubes) and the assay was carried out in accordance with the method of Tice et al. [31] and OECD guideline 489 [32]. The liver tissues were obtained after sacrificing the animals. In the liver, the comet assay was performed as per the method of Miyamae et al. [33]. The tissues were minced and suspended in chilled homogenising buffer (100 mg/ml, pH 7.5). The samples were homogenized gently with Miccra D-1 high-speed tissue homogeniser (400–600 rpm). Cell viability was determined by the trypan blue exclusion assay [34]. Peripheral blood leukocytes (PBL) and liver homogenates were diluted separately with PBS and mixed with 0.4% trypan blue dye. The cells with intact membrane were viable and excluded the dye, and they were counted with a haemocytometer. For each tissue three slides were prepared. In brief, the slides were coated with a layer 100 µl of 1% NMA in PBS, then covered with a coverslip and allowed to solidify overnight at 37 °C. For the preparation of the second layer, 20 µl of whole blood or liver (1–5 × 10^4 cells) homogenate was suspended in 100 µl of 0.5% LMA. The suspended cells and LMA were placed on the pre-coated slides and spread uniformly and covered with a coverslip. Then the slides were dried at 4 °C for ten minutes. The third layer of plain 0.5% LMA (120 µl) was placed on the slides, and a coverslip was quickly put to get an even layer and dried at 4 °C. 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 after removing the coverslips. The slides were pre-soaked for 20 min in alkaline buffer (10 M NaOH, 200 mM Na_2 EDTA, pH > 13.0). 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. The coded slides were stained with 20 µg/ml of ethidium bromide and examined using a fluorescence microscope with a blue excitation filter (488 nm) and yellow emission (barrier) filter (515 nm) at × 400 magnifications (Olympus, Shinjuku-ku, Tokyo, Japan). One hundred and fifty cells were randomly selected per rat (50 cells per slide) and used to document the DNA damage. The damage was expressed as percentage of DNA in the comet tail. DNA breakage was measured by using a version Komet 5.5 Comet Image Analysis System (Single Cell Gel Electrophoresis analysis company, Andor Technology 2005, Kinetic Imaging Ltd., Nottingham, UK).

2.5.2. Micronucleus test

The MNT was executed in bone marrow after 24 h of repeated oral exposure with WO_3 NPs and MPs in rats as per OECD guideline 474 with slight modifications [35]. The bone marrow cells were extracted from thigh bones by aspiration. The extractions were placed in hypotonic solution made of 1% sodium citrate and centrifuged for 5 min at 1000 rpm. The cell pellet was resuspended in 1% sodium citrate, and a smear was prepared on microscopic slides and dried overnight in humidified air. The slides were immersed in methanol (fixative) for 2 min and stained with 0.5% Giemsa stain for 3 min. The slides were then examined at ×1000 magnification for appearance of MN. For each rat, three slides were prepared. Randomly two thousand polychromatic erythrocytes (PCEs) per animal from each slide and the frequency of micronucleated PCEs (MN-PCEs) were noted. The PCEs to normochromatic erythrocytes (NCEs) in the bone marrow, thousand cells from each animal were examined and the ratio was expressed as percentage: (PCEs × 100/PCEs + NCEs).

2.6. Histopathological evaluation

Various tissues like brain, heart, kidney, liver, and spleen of albino Wistar rats after 28 days of repeated oral dose of WO_3 NPs and MPs were examined for histopathological damage. The tissues were fixed in formalin after sacrifice. They were processed, embedded in paraffin blocks, sliced into 3-µm thick sections and mounted on slides. Tissues were stained with haematoxylin and eosin (H & E) and mounted with DPX. A Nikon Eclipse E 800 microscope (Nikon Eclipse E 800, Japan) was used to scrutinize the slides at × 400 magnification. Three random sections per slide were assessed for histopathological changes.

2.7. Biochemical assays

The whole blood was collected from all the rats by puncturing the retro-orbital plexus and separated batch wise. For hematology parameters, the blood was collected in EDTA-coated tubes. For biochemical estimations, the samples without using anticoagulant were kept for 45 min at room temperature. The serum obtained from the blood was centrifuged at 2500 rpm for 15 min at room temperature and used for the determination of AST and ALT levels. The liver tissues were collected after sacrificing the animals which were used to analyse the reduced GSH content, catalase and lipid peroxidation. The tissues were quickly dissected, washed in ice-cold normal saline and then with buffer of pH 7.4 (0.15 M Tris-HCl), dried and weighed. Liver tissues from each group were then homogenized independently in ice-cold sucrose (0.25 M) to make 10% homogenate (w/v) with a Miccra D-1 high speed tissue homogenizer. To remove debris, the homogenate was centrifuged for 10 min at 10,000 × *g* at 4 °C. All the biochemical indices were estimated after 28 days repeated oral treatment with 250, 500 and 1000 mg/kg bw/day doses of WO_3 NPs and MPs using a spectrophotometer (Spectramax Plus, Molecular Devices, USA).

2.7.1. Hematological estimation

The blood parameters such as red blood cells (RBC), white blood

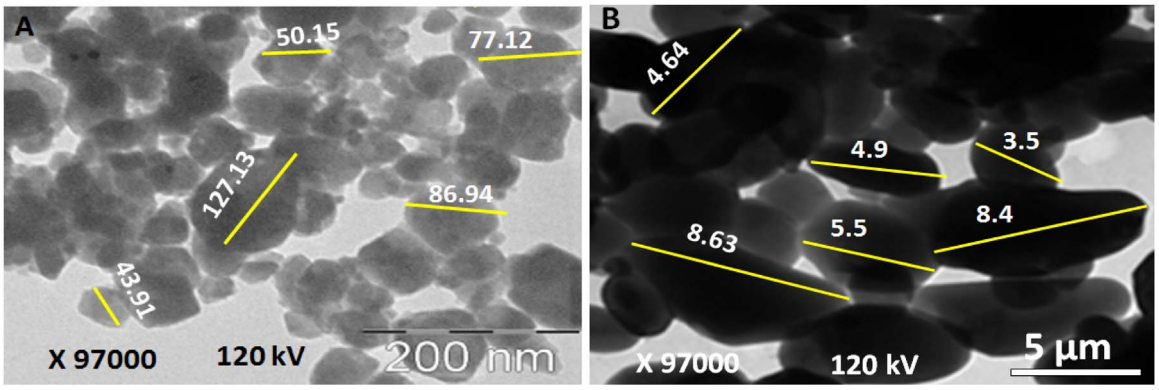

Fig. 1. TEM image of (A) WO_3 NPs and (B) WO_3 MPs in Milli-Q water for characterisation of particles. Particles were scanned by TEM at 120 kV and a resolution of x 97000. 100 particles of measurements were calculated from random fields of view and images showing the general morphology.

cells (WBC), haemoglobin (HGB), hematocrit (Hct) and mean corpuscular volume were determined with a hematology analyser ABX Micros ES 60 (HORIBA Medical, France).

2.7.2. AST and ALT activity

Activity of AST and ALT enzyme levels were measured according to procedure of Yatzidis et al. [36]. 0.05 ml of serum was added to 0.5 ml of substrate (AST and ALT in phosphate buffer pH 7.4) and incubated at 37 °C for an hour in water bath. After incubation, 0.5 ml of aniline citrate and 0.5 ml of 2,4-Dinitrophenylhydrazine (DNPH) were added to above solution and shaken vigorously to terminate the reaction and kept for 5 min at room temperature. Subsequently, 3 ml of 0.75 M NaOH was added quickly. The solution was incubated for 30 min and the absorbance was read at 505 nm. AST and ALT enzyme levels were expressed as µmol/h/ml in serum.

2.7.3. Reduced glutathione content

The GSH content was analysed in the liver tissue according to the protocol of Jollow et al. [37]. One gram of liver tissue was rinsed in physiological saline (ice cold), perfused with cold KCl buffer (1.15% KCl and 0.5 mM EDTA) and homogenized in potassium phosphate buffer (0.1 M, pH 7.4, KPB) with a homogenizer. An aliquot of 0.5 ml of the tissue homogenate was mixed with 0.5 ml of sulfosalicylic acid (4% w/v) and incubated for 1 h on ice and centrifuged at 10,000 rpm for 10 min 0.4 ml of supernatant was added to 0.4 ml of DTNB (4 mg/ml in 5% sodium citrate) and 2.2 ml KPB (0.1 M, pH 7.4) was mixed with it. The yellow colour, thus observed was read at 412 nm. The quantity of GSH present was expressed as µg GSH/g wet weight of tissue.

2.7.4. Catalase

Catalase activity in blood was determined by the procedure of Sinha [38]. 100 µl of 10% liver homogenate was added to 0.5 ml of H_2O_2 (0.2 M) and incubated at 37 °C for 90 s with 0.01 M phosphate buffer (pH 7.4). Reaction was terminated by addition of 5% dichromate solution. Then the solution was incubated for 15 min at 100 °C for in a water bath. Quantity of H_2O_2 consumed was measured by the absorbance at 570 nm. The enzyme activity was expressed as U/mg protein.

2.7.5. Lipid peroxidation

Malondialdehyde (MDA) which is the end product of lipid peroxidation was measured in liver tissue homogenate according to the procedure of Ohkawa [39] with slight modifications. A 200 µl aliquot of the tissue homogenate was mixed with 2 ml of 0.375% of thiobarbituric acid (TBA) and 15% of trichloroacetic acid (TCA) reagent and made up to 3 ml with distilled water. The mixture was incubated in a water bath at 95 °C for 20 min and cooled under tap water. To the solution, 3 ml of n-butanol was added to obtain the reaction product (TBA–MDA complex). The absorbance of the end product (pink colored) was estimated at 532 nm. The quantity of MDA was determined using a molar extinction coefficient of 1.56×10^5 M/cm and expressed as nmoles of MDA formed per gram wet weight of the tissue.

2.8. Tungsten content in different tissues, blood, urine and feces

Throughout the 28 days treatment, urine and feces specimens were collected daily. After the termination of exposure period the various tissues were obtained for W content analysis. The samples were processed in accordance with the procedure of Gomez et al. [40]. The samples were digested in nitric acid for 12 h, and then heated at 80 °C for 10 h initially. Then for 30 min they were heated at 130–150 °C. To the samples, 0.5 ml of 70% per chloric acid was added. The samples were heated again for 4 h for drying. For measuring the W content, samples were filtered and 2% of HNO_3 was added to make final volume of 5 ml. The W standard solution was diluted (serially) to 1, 10, 50, 100 ppm. The content of W in the samples was evaluated by ICP-OES (JY Ultima, Jobin Vyon, France).

2.9. Statistical analysis

The significant alterations between treated and control groups were statistically analysed by one-way ANOVA. The results obtained were represented as mean and standard deviation (mean ± SD) of the mean. The Dunnett's multiple comparison post-test was used for multiple pairwise comparisons to confirm the significance of positive response. Statistical analysis was conducted using GraphPad Instat Prism 3 Software package for Windows (GraphPad Software, Inc., La Jolla, CA, USA). Significance for all tests was set at minimum of $p < 0.01$.

3. Results

3.1. Characterization of WO_3 NPs and MPs

The mean size of WO_3 NPs and MPs was 52 ± 2.97 nm, and 5.73 ± 7.58 µm and the morphology was spherical for both the particles using TEM. The images obtained are given in Fig. 1(A & B). WO_3 NPs showed a size of 195.6 nm in Milli-Q water suspension when measured by DLS. WO_3 NPs showed larger values with DLS in comparison to TEM measurements, indicating that WO_3-NPs formed larger agglomerates in water suspension. Electrophoretic Mobility and Zeta potential measurements using LDV were −0.75 and −6.03 mV (µm cm/V/s) respectively, at pH 7.0. In the case of WO_3 MPs, DLS and LDV data were found to be out of detection limit (Table 1). The crystal structure of the NPs and MPs was hexagonal and tetragonal, respectively by XRD and is presented in Table 1 and Fig. 2A & 2B. The specific surface area of WO_3 NPs and MPs was 19.33 and 15.15 (m^2/g) determined through BET analysis. The results of purity analysis showed the presence of < 0.92% impurity in WO_3 NPs and < 1.23% of

Table 1
Characterization of WO_3 NPs and MPs.

Particles	Size using TEM (nm)	Shape	DLS		LDV			Crystalline structure	SSA (m^2/g)	Purity analysis (ICP-OES)
			Average diameter (nm)	PDI	Zeta potential ζ (mV)	Electrophoretic Mobility (μm·cm/V/s)	pH			
WO_3 NPs	52 ± 2.97(nm)	Sphere	195.6	0.411	−6.03	−0.75	7.0	Hexagonal	19.33	> 99.08
WO_3 MPs	5.73 ± 7.58(μm)	Sphere	ND	ND	ND	ND	7.0	Tetragonal	15.15	> 98.77

DLS = Dynamic Light Scattering, PDI = Polydispersity Index, LDV = Laser Doppler Velocimetry, SSA = Specific surface area, ND = not detectable.

contamination in WO_3 MPs. Hence the purity of WO_3 NPs was > 99.08% and of WO_3 MPs was > 98.77%.

3.2. Animal observation, food consumption, body weight and organ weight

Mortality, adverse signs or symptoms were not observed after 28-day repeated oral doses of 250, 500, 1000 mg/kg bw/day of WO_3 NPs and MPs in male and female Wistar rats. However, rats administered with the highest dose of NPs were irritated, dull and showed moribund symptoms during the first week of treatment. Further, both NP and MP-treated rats showed a little loss in body weight (Fig. 3A) and feed intake (Fig. 3B). Moreover, the relative organ weights of all the tissues from both the sexes of rats were slightly increased when compared to tissues of control rats (Fig. 3C).

3.3. Genotoxicity analysis

3.3.1. Comet assay

Cell viability of PBL and liver cells of all the samples ranged from 87 to 95%. There was no significant difference between PBL and liver cells of all the samples when compared to respective controls (data not shown). The results obtained by comet assay after 28 days repeated oral administration of the doses of 250, 500, 1000 mg/kg bw/day in PBL and liver when treated with WO_3 NPs and MPs in male and female Wistar rats are shown in Fig. 4(A & B). The data obtained suggested that there was a significant ($p < 0.01$) increase in the DNA damage (% tail DNA) in the PBL and liver of rats exposed to WO_3 NPs at the dose of 1000 mg/kg bw/day in both the sex of rats. A statically insignificant enhancement in% tail DNA was also observed in the PBL and liver with 250, 500 mg/kg bw/day dose of NPs and all the doses of MPs when compared to the control group. The positive control (CP) rats showed a significantly higher mean% of tail DNA ($P < 0.001$) compared with the control animals. Moreover, gender-dependent variation in% of tail DNA was not apparent.

3.3.2. Micronucleus test

The bone marrow MNT was also conducted after 28-day repeated oral treatment with 250, 500 and 1000 mg/kg bw/day of WO_3 NPs and MPs in rats. The mean frequency of MN-PCEs in bone marrow cells of rats orally exposed to 1000 mg/kg bw/day dose of NPs was observed to be significantly increased ($P < 0.01$) in a gender-independent manner compared to control. However, WO_3 NPs at 250, 500 mg/kg bw/day and MPs at all doses showed an insignificant increasing trend in the mean frequency of MN PCEs in bone marrow cells of rats in comparison to the control (Table 2). The group treated with CP produced a markedly significant ($P < 0.01$) effect on MN-PCEs frequency. There was a no significant decrease in% PCEs in bone marrow cells of both genders in comparison to the control animals.

3.4. Hematological estimations

The hematological results revealed that HGB, RBC, PLT and HCT values of the WO_3 NP and MP-treated male and female rats were reduced, while WBC of these rats was elevated with increasing doses of WO_3 NPs and MPs compared to control groups of both the sex of rats. Slight significant differences were observed ($p < 0.01$) when treated with WO_3 NPs at 1000 mg/kg bw/day compared with 250, 500 mg/kg bw/day of WO_3 NPs and all the doses of WO_3 MPs (Table 3).

3.5. Biochemical alterations

The repeated doses of WO_3 NPs significantly increased the AST and ALT levels in serum at the dose of 1000 mg/kg bw/day ($P < 0.01$) whereas with WO_3 NPs at 250, 500 mg/kg bw/day and MPs of all the doses, the enzyme levels were slightly increased in a dose-dependent manner, but the increase was insignificant in both the sex of rats when compared to control group (Table 4). Moreover, the gender difference was not an apparent. The level of MDA, which is the end product of lipid peroxidation, was significantly increased, and the GSH and CAT levels were significantly decreased when treated with the dose of

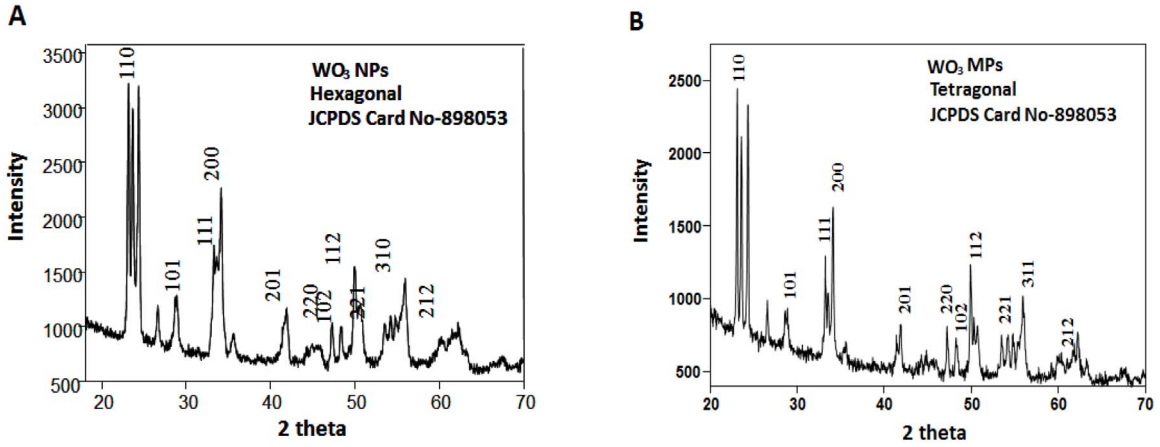

Fig. 2. XRD Patterns of (A) WO_3 NPs (Hexagonal) and (B) WO_3 MPs (Tetragonal). The scan parameters were set at a scan rate of 1.2° per minute and scan range was 2θ = 0–80°. All the reflection peaks corresponded to the facets of face centred cubic structure of WO_3 Particles and the peaks were consistent with standard database files (JCPDS card No. 898053).

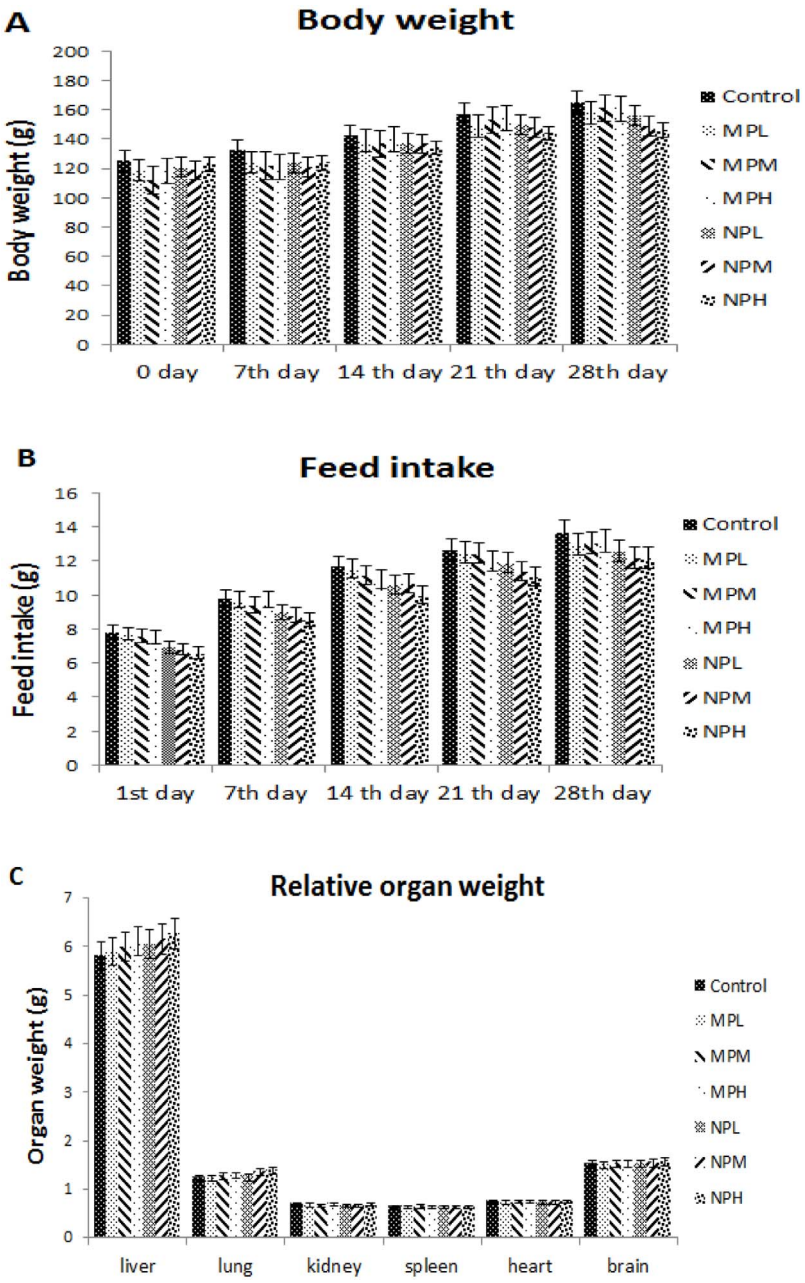

Fig. 3. Effect of WO_3 NPs and MPs on body weight (A), feed intake (B), and relative organ weight (C) profile. Data represent mean weight ± SD, n = 5 animals per group. MP = Microparticle, NP = Nanoparticle, L = Lower dose, M = Medium dose, H = Higher dose. MPL = 250 mg/kg bw/day, MPM = 500 mg/kg bw/day and MPH = 1000 mg/kg bw/day of WO_3 MPs. NPL = 250 mg/kg bw/day, NPM = 500 mg/kg bw/day, NPH = 1000 mg/kg bw/day of WO_3 NPs.

1000 mg/kg bw/day of WO_3 NPs in male and female rats compared to control rats. At doses of 250 and 500 mg/kg bw/day of WO_3 NPs and all the doses of MPs, an enhancement in the level of MDA and the slight decrease in the levels of GSH and CAT were observed, which were statistically insignificant compared to control group of rats.

3.6. Histopathological examinations

The slides of tissues namely liver, kidneys, spleen, heart and brain stained with H & E were studied. All the tissue sections were evaluated for any toxic changes. Focal areas of necrosis (FAN) were observed in the liver after exposure with 1000 mg/kg bw/day dose of WO_3 NPs in female and male rats when compared to control group (Fig. 5B). However, no morphological changes were observed in the tissues of kidney, spleen, heart and brain treated with WO_3 NPs at the higher dose. Further, there were no apparent effects recorded at 250 and 500 mg/kg bw/day dose groups of WO_3 NPs. Similarly, significant morphological changes were not found in the liver, kidneys, spleen, heart and brain of WO_3 MP-treated rats at all the doses (data not shown).

3.7. Biodistribution of WO_3 NPs and MPs

Repeated oral exposure of WO_3 NPs and WO_3 MPs for 28 days with 250, 500 and 1000 mg/kg bw/day doses in male and female Wistar rats was carried out by ICP OES. The results revealed a statistically significant increase in W concentration, indicating that accumulation had occurred in tissues. W content was significantly accumulated in the liver > lungs > kidney > blood > spleen > heart at the experimental doses of 500 and 1000 mg/kg bw/day of WO_3 NPs when compared to their MPs in both the sexes. Values for the accumulation and excretion patterns (µg/g and µg/ml) are given in Fig. 6A-I.

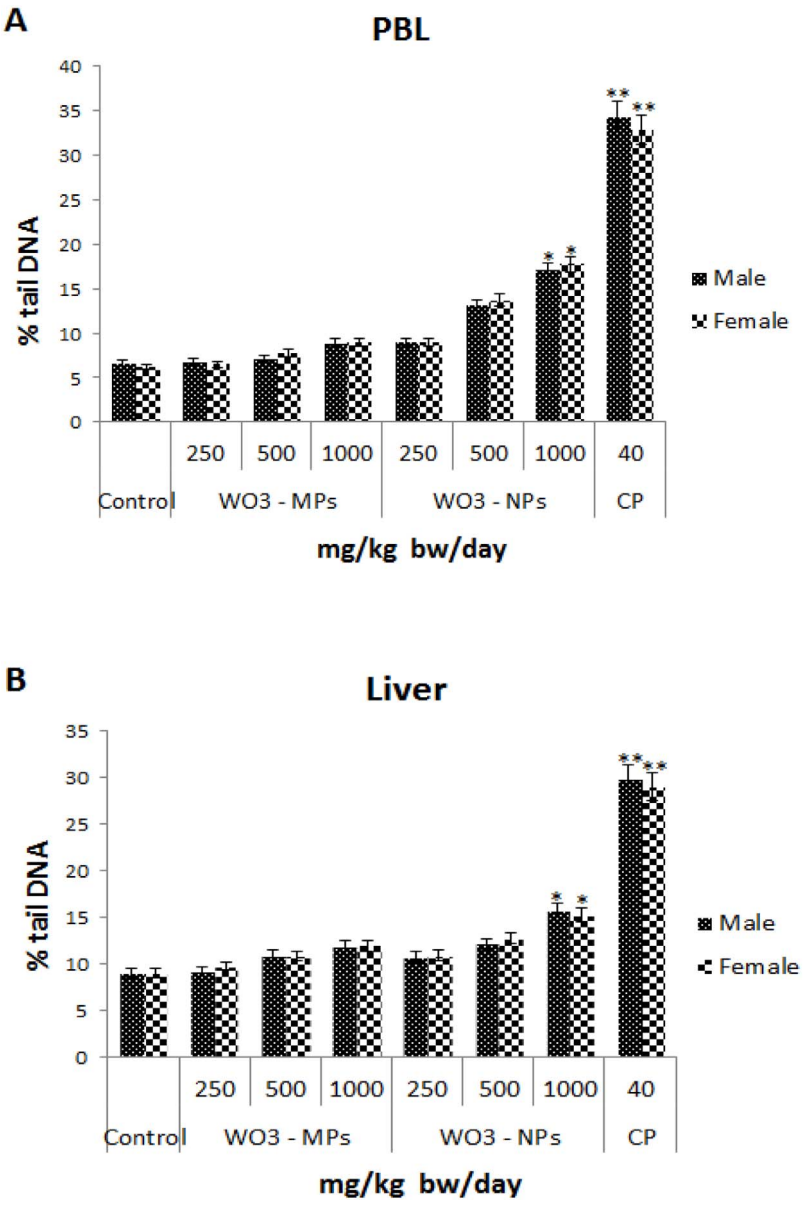

Fig. 4. Mean% tail DNA in PBL (A) and liver (B) of male and female Wistar rats after 28 days of repeated study with different oral doses of WO_3 NPs and MPs. Milli-Q water (control) and CP (positive control). Data represented as mean ± SD, significantly different from control at $^*P < 0.01$, $^{**}P < 0.001$, n = 5 animals per group.

Table 2
The frequency of MN-PCEs and % PCEs in male and female Wistar rat bone marrow cells treated orally after 28-day repeated exposure of WO_3 NPs and MPs.

Treatments	Dose (mg/kg bw/day)	Male		Female	
		MN-PCEs	%PCEs	MN-PCEs	%PCEs
Control[a]	Milli-Q water	4.14 ± 0.13	41.17 ± 1.1	4.2 ± 1.14	40.99 ± 1.04
WO_3 NPs	250	4.4 ± 1.92	38.77 ± 1.2	4.6 ± 1.21	39.13 ± 0.14
	500	5.6 ± 1.71	36.89 ± 0.45	5.77 ± 2.6	37.70 ± 1.2
	1000	6.98 ± 1.12*	33.37 ± 0.5	7.19 ± 3.91*	33.00 ± 1.6
WO_3 MPs	250	4.03 ± 1.1	39.07 ± 0.2	4.04 ± 1.67	39.89 ± 0.5
	500	5.17 ± 1.14	38.83 ± 0.63	5.43 ± 2.01	38.24 ± 0.6
	1000	5.8 ± 2.28	37.81 ± 0.17	5.96 ± 2.86	36.36 ± 0.02
CP[b]	40	33.2 ± 3.13**	23.40 ± 0.2	32.6 ± 3.66**	23.03 ± 0.19

Data represented as mean ± S.D. Significantly different from control at $^*P < 0.01$, $^{**}P < 0.001$, n = 5 animals per group, [a]Negitive control (Milli-Q water), [b]Positive control (40 mg/kg bw/day)

Table 3
Hematological parameters in rats by oral administration daily for 28 days with WO_3 NPs and MPs.

Treatments	Dose (mg/kg bw/day)	Male					Female.				
		HGB (g/dl)	RBC (10^{12}/L)	WBC (10^9/L)	PLT(10^9/L)	HCT (%)	HGB (g/dl)	RBC (10^{12}/L)	WBC (10^9/L)	PLT(10^9/L)	HCT (%)
Control[a]	–	14.13 ± 0.21	8.04 ± 0.11	10.76 ± 0.2	532.42 ± 0.1	40.1 ± 0.31	13.84 ± 0.1	7.99 ± 0.31	10.1 ± 0.12	520.3 ± 0.12	39.1 ± 0. 17
WO_3 NPs	250	14.01 ± 0.11	7.83 ± 0.41	10.99 ± 0.12	524.15 ± 0.13	39.04 ± 0.14	13.01 ± 0.71	7.81 ± 0.23	10.87 ± 0.32	510.7 ± 0.71	38.34 ± 0.5
	500	13.1 ± 0.7	7.13 ± 0.12	11.82 ± 0.71	511.12 ± 0.4	38.1 ± 0.11	12.13 ± 0.4	7.23 ± 0.12	11.77 ± 0.61	500.20 ± 0.14	37.7 ± 0.17
	1000	12.04 ± 0.32*	6.34 ± 0.33*	12.96 ± 0.15*	482.23 ± 0.13*	37.10 ± 0.24*	11.74 ± 0.16*	6.51 ± 0.41*	12.79 ± 0.15*	491.01 ± 0.11*	36.97 ± 0.74*
WO_3 MPs	250	14.11 ± 0.31	8.01 ± 0.29	10.86 ± 0.13	529.49 ± 0.17	39.79 ± 0.12	13.54 ± 0.1	7.96 ± 0.2	10.16 ± 0.35	515.20 ± 0.14	39.09 ± 0.25
	500	13.96 ± 0.35	7.82 ± 0.17	11.31 ± 0.31	522.32 ± 0.11	39.01 ± 0.42	12.97 ± 0.15	7.87 ± 0.13	10.931 ± 0.81	509.12 ± 0.2	38.74 ± 0.43
	1000	13.52 ± 0.2	7.63 ± 0.14	12.06 ± 0.23	517.4 ± 0.9	38.71 ± 0.71	12.67 ± 0.14	7.79 ± 0.4	11.77 ± 0.53	503.41 ± 0.11	37.82 ± 0.31

Values represent means ± SD. [a]Control (Milli-Q water), significantly different from control at $*P < 0.01$. n = 5 animals per group.

Table 4
Effects of WO_3 NPs and MPs in serum and liver biochemical and oxidative stress parameters in male and female Wistar rats treated orally after 28-day repeated exposure.

Treatments	Dose (mg/kg bw/day)	Male					Female				
		AST[b] (μmol/h/ ml)	ALT[b] (μmol/h/ ml)	GSH[c] (μg/g tissue)	Catalase[c] (U/mg protein)	MDA[c] (nmol/g)	AST[b] (μmol/h/ ml)	ALT[b] (μmol/h/ ml)	GSH[c] (U/mg protein)	Catalase[c] (U/mg protein)	MDA[c] (nmol/g)
Control[a]	–	7.17 ± 1.89	5.3 ± 1.04	7.1 ± 0.89	190.04 ± 1.7	5.54 ± 1.59	7.01 ± 1.04	5.21 ± 1.04	6.91 ± 0.189	189.4 ± 0.87	5.51 ± 1.1
WO_3 NPs	250	7.87 ± 0.14	5.85 ± 1.2	6.16 ± 0.1	165.1 ± 0.1	6.25 ± 1.24	7.95 ± 0.63	5.91 ± 0.3	6.19 ± 0.36	161.8 ± 1.4	6.05 ± 0.4
	500	8.92 ± 0.25	6.2 ± 0.87	4.87 ± 0.23	134.5 ± 0.5	6.59 ± 1.89	8.88 ± 0.32	6.18 ± 0.12	4.79 ± 0.51	134.1 ± 1.7	6.65 ± 0.52
	1000	9.91 ± 1.1*	6.95 ± 1.8*	3.68 ± 0.1*	105.1 ± 0.6*	7.23 ± 0.45*	9.85 ± 0.2*	6.89 ± 0.7*	3.89 ± 0.*	100.85 ± 0.9*	7.3 ± 0.7*
WO_3 MPs	250	7.31 ± 0.85	5.78 ± 0.9	6.89 ± 0.4	178.5 ± 0.84	5.41 ± 0.36	7.0 1 ± 0.23	5.0 1 ± 0.3	6.09 ± 0.6	182.51 ± 0.4	5.01 ± 1.1
	500	7.55 ± 1.89	5.81 ± 1.21	5.58 ± 0.5	170.75 ± 0.11	5.99 ± 0.14	7.37 ± 0.74	5.87 ± 0.74	5.57 ± 0.7	168.7 ± 1.8	5.73 ± 0.8
	1000	8.12 ± 0.8	6.16 ± 2.14	5.26 ± 1.7	163.3 ± 1.6	6.28 ± 0.17	8.15 ± 0.4	6.25 ± 0.4	5.17 ± 0.63	161.3 ± 2.1	6.08 ± 0.63

Data represented as mean ± S.D. [a]Control (Milli-Q water), [b]serum, [c]liver. Significantly different from control at $*P < 0.01$, n = 5 animals per group.

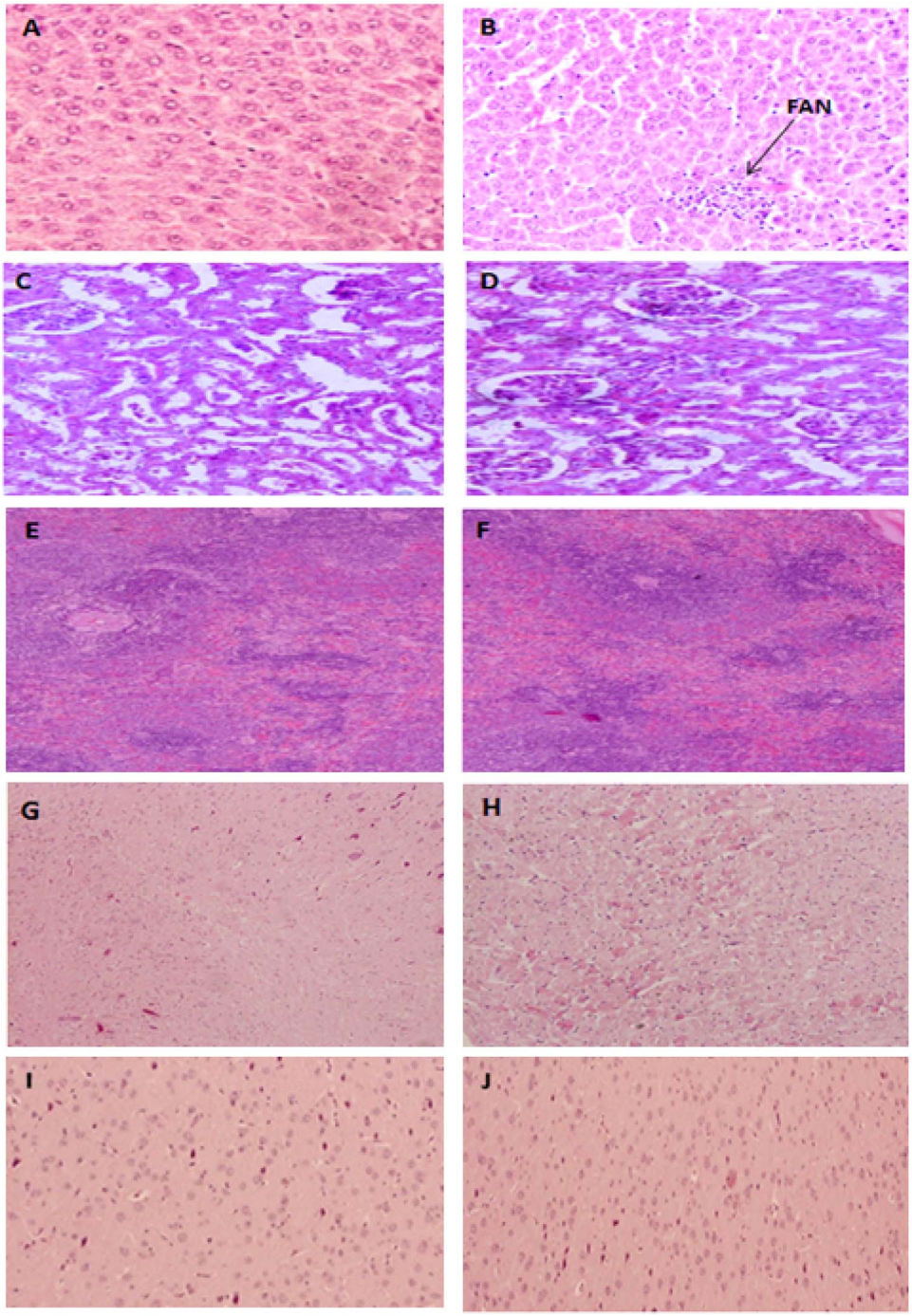

Fig. 5. Histopathology of liver, kidney, spleen, heart and brain tissues of female rats after 28 days of repeated oral treatment with WO_3 NPs at 1000 mg/kg bw/day. Normal architecture of liver (A), kidney (C), spleen (E), heart (G) and brain (I) in control rat tissues. Figure (B) of liver showing the FAN (focal areas of necrosis) followed by kidney (D), spleen (F), heart (H) and brain (J) images showing no morphological changes were observed in treated tissues.

Additionally, the data was normalised with respect to the average rat weight for the increase of dosed WO_3 NPs and WO_3 MPs with increased weight. Further, the percentage of W recovered in each tissue, whole blood, urine and feces from the administered doses are shown in Table 5. The distribution of W content was maximum at 1000 mg/kg bw/day followed by 500 and 250 mg/kg bw/day in male and female rats treated with WO_3 NPs and MPs in a dose dependent and gender-independent manner. However, the distribution of W in various tissues was significantly higher in the NP-treated groups as compared to MP-treated groups at all dose levels. Moreover, significant amount of W was excreted in the urine at 1000 mg/kg bw/day of NP-dosed rats when compared MPs. In contrast, with MP-treated rats more significant excretion of W content in the feces was observed when compared to NP-treated rats

4. Discussion

The present study was executed to provide the first report of the genotoxicity, hematology, biochemical alterations and biodistribution of the W in different tissues, whole blood, urine and feces after 28 days of repeated oral administration with 250, 500 and 1000 mg/kg bw/day doses of WO_3 NPs and MPs in albino Wistar male and female rats. The results obtained have revealed that the WO_3 NPs induced toxic effects at high dose without any severe distress symptoms and mortality. In

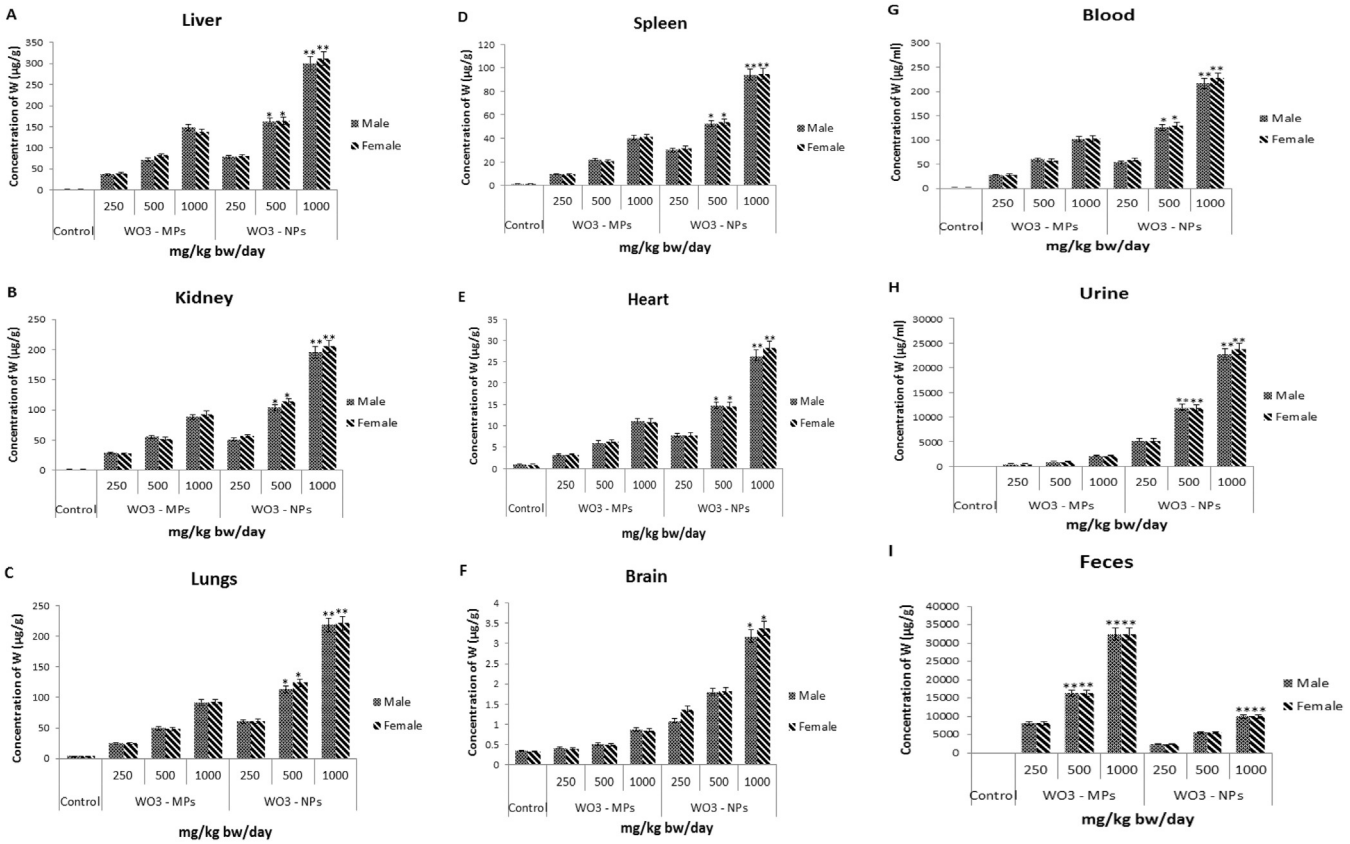

Fig. 6. Tissue distribution of W measured by ICP-OES in (A) liver, (B) kidneys, (C) lungs, (D) spleen, (E) heart, (F) brain, (G) blood (H) urine and (I) feces of rats after 28 days of repeated oral administration with 250, 500 and 1000 mg/kg bw/day doses of WO_3 NPs and MPs. Control (Milli-Q water). Significantly different from control at $^*P < 0.01$ and $^{**}P < 0.001$, n = 5 animals per group.

Table 5
The percentage (%) of W recovered in liver, kidney, lungs, spleen, heart, blood, urine and feces from the various administered doses of WO_3 NPs and MPs in male and female rats after 28 days repeated oral exposure.

Treatments	Dose (mg/kg bw/day)	Male (% of the administered doses)									Female (% of the administered doses)								
		Liver	Kidney	Lungs	Spleen	Heart	Brain	Blood	Urine	Feces	Liver	Kidney	Lungs	Spleen	Heart	Brain	Blood	Urine	Feces
Control[a]	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–
WO_3 NPs	250	1.25	0.202	0.242	0.060	0.015	0.005	0.70	50.14	21.05	1.28	0.225	0.242	0.063	0.012	0.005	0.78	50.12	21.00
	500	1.29	0.209	0.232	0.058	0.014	0.004	0.83	55.20	25.69	1.31	0.226	0.245	0.053	0.014	0.005	0.86	55.48	25.66
	1000	1.30	0.207	0.235	0.052	0.014	0.003	0.72	56.20	25.74	1.34	0.217	0.236	0.050	0.014	0.003	0.75	55.51	25.79
WO_3 MPs	250	0.59	0.112	0.100	0.018	0.006	0.001	0.36	5.14	75.18	0.62	0.108	0.108	0.019	0.006	0.001	0.37	5.12	75.13
	500	0.55	0.110	0.108	0.020	0.005	0.001	0.39	5.20	75.85	0.65	0.104	0.104	0.020	0.005	0.001	0.38	5.41	75.80
	1000	0.52	0.087	0.103	0.021	0.005	0.0008	0.33	6.20	75.99	0.59	0.091	0.103	0.020	0.004	0.0003	0.38	6.91	75.84

[a] Control (Milli-Q water).

contrast, MPs did not show toxicity at all the test concentrations. Probably, because of their small size, crystalline structure and large surface area, the NPs were able to enter easily into the body via oral route than the MPs. Thus, WO_3 NPs can be classified as materials with toxic effects when exposed to higher doses for a long time. Moreover, the physico-chemical properties of the NPs could have influenced the NP behaviour and may have more impact on toxicity than the MPs. A few earlier studies have shown that NPs are more toxic than their MPs via the oral route [15,41–45].

In the current investigation, comet assay showed that WO_3 NPs were able to induce significant% tail DNA in PBL and liver at the higher dose as compared to the control. Similar *in vivo* genotoxicity studies with WO_3 NPs using comet assay have not been reported. However, other metal oxide NPs are available which are in line with our comet assay results [35,44–46]. The bone marrow MNT results revealed a significant increase in MN frequency at 1000 mg/kg bw/day dose of WO_3 NPs. Even though, the tested higher dose (1000 mg/kg bw/day) of WO_3 NPs for MN reached statistically significance, the NPs may still be considered as a weak genotoxicant. The results suggest that either clastogenic or aneugenic event may be involved in the formation of MN with WO_3 NPs. The% PCEs induced a slight decrease compared with the control group, suggesting that cell death had occurred in all the treated groups. Similar results were obtained with Cr_2O_3, CeO_2 and MnO_2, NPs after 28-day oral administration [35,44,45]. Sycheva et al. [47] found that after exposure of TiO_2 NPs to 1000 mg/kg bw/day by oral route may lead to a significant increase in MN-PCE frequency and DNA damage in bone marrow cells of male CBAB6F1 mice.

The hematological examination of WO_3 NP- treated rats showed a slightly significant decrease in HGB, RBC, PLT and HCT and a marginally significant increase in WBC at 1000 mg/kg bw/day dose only.

Similar results were also observed in mice and rats with TiO2 NPs [8,48]. The reduction in RBC, HCT and HGB might be due to the inhibition of heme synthesis and erythropoiesis and to an enhancement in the pace of erythrocyte loss in the hemopoietic organs. In this study, the WO_3 NP-treated animals also exhibited significantly higher WBC when compared to the control animals. The increase in WBC count suggested that the defence system was activated in the body [49]. Liver function was determined in the current study by serological markers such as AST and ALT. WO_3 NPs significantly increased the activities of AST and ALT enzymes in the serum of animals administered with 1000 mg/kg bw/day, suggesting probable injury in the tissues. Moreover, the gender difference was not apparent, and exposure was in a dose-dependent manner. Similar results were observed in rats treated with three different NPs (MnO_2, ZnO and Fe_2O_3) after 28 days of oral administration [45,46,50].

The present study also focused on the oxidative effects induced by WO_3 NPs and MPs. Oxidative stress and free radicals can induce damage to lipids and cell membranes, causing cell apoptosis or death. Therefore, in the current study, the levels of MDA, CAT and GSH were estimated in liver tissue. Significant increase in MDA and depletion in GSH, as well as CAT levels were observed in the liver at 1000 mg/kg bw/day dose of WO_3 NPs indicating induction of free radical generation. When excessive reactive oxygen species (ROS) are generated; the levels of MDA may be enhanced, and the GSH and CAT levels could be reduced, suggesting that the WO_3 NP-treated animals suffered oxidative stress situation. Investigations with Fe_2O_3 and CeO_2 NP-treated rats also revealed a similarly significant effect in MDA levels, decrease in GSH content and CAT activity in the liver [44,51]. Additionally, the histopathological examination may be helpful in detecting an abnormality in tissues of rats. In the current study, rats treated with 1000 mg/kg bw/day dose of the WO_3 NPs for 28 days showed focal areas of necrosis (FAN) in the liver of both sexes. Similar changes were documented with other metal oxide NPs [44–46,50].

The biodistribution study revealed that W ions from NP or MP-treated rats could pass across the gastrointestinal barrier and accumulate in the organs and tissues. The majority of W content from NP-treated rats was found in the liver, blood, kidney as well as spleen. The excretion data showed that a higher quantity of W content was excreted via urine after NPs administration, whereas significant amounts of W were excreted via feces when treated with MPs. Our study demonstrated that in comparison to NPs, a lesser amount of W content of MP treated rats could pass through the intestinal barrier, and large quantities were quickly excreted via feces. Some studies with other NPs and MPs (Cr_2O_3, CeO_2, MnO_2 and Cu) are also with the concurrence of our findings [35,44,45,52]. Collectively, a significant recovery of 2.61% of W content was found in the tissues of rats whereas 77.9% was excreted via urine and feces when treated with WO_3 NPs. In the case of WO_3 MPs, 1.24% the W content was found in the tissues, and 81.4% was excreted during the full term of this repeated oral dose study when compared to control group of both sexes. Moreover, a significant difference of more than 2 fold in the accumulation of W in the tissues of WO_3 NP-treated rats was observed when compared to MPs in both genders. The body burden was comparatively small when considering the total dose administered over the repeated oral study, suggesting that the majority of administered W content was eliminated. However, there is a chance for the accumulation of W in various other tissues such as bone marrow, femur, stomach, GI tract, large and small intestines which were not evaluated in this study. Additional studies with all the tissues and more post-dose time points are needed to fully characterise the kinetics of W after administration with WO_3 NPs and WO_3 MPs.

The liver is one of the first organs responsible for detoxification and to suffer damage when an animal is exposed to foreign substances [53]. The oral exposure to WO_3 NPs led to liver damage which was revealed by the comet assay with an increase in% tail DNA and hepatocellular necrosis in the liver tissue. These results were also supported by the elevated AST and ALT enzyme levels in the serum indicating that enzymes rise in serum when the liver is damaged. The proposed mechanism responsible for the accumulation of WO_3 NPs in the liver may be activation of metallothioneins involved in detoxification of heavy metals, including W [54]. Moreover, high W deposition in the liver may be associated with adequate filtration and the presence of specific subpopulations of mononuclear phagocytic cells. These phagocytes are part of the reticulo-endothelial system (RES), which takes part in sequestration of various NPs. The elements of RES in the liver are Kupffer cells are located in specific sinusoidal walls, so they can remove impurities from the blood efficiently. Reports showed that NPs are taken by Kupffer cells via endocytosis [55–58]. Cellular uptake of WO_3 NPs and MPs using TEM could have provided the confirmatory evidence of the particles entering into the tissues. Although we did not study the entry mechanism of WO_3 particles, it is reasonable to suppose that the results of the current study are in concurrence with the findings of Sharma et al. [46] who observed liver damage in mice after repeated oral exposure to ZnO NPs at the higher dose of 300 mg/kg.

Our results showed that the toxicology of WO3 NPs was size and dose dependent. The size of particles has proven to be very influential to their behaviour

It is suggested that NPs have a broader tissue distribution, pass into the body of the animals and accumulate to a larger proportion, have a greater toxicity potential than their MPs. Limited studies have compared NPs and MPs often. Therefore the inclusion of an MP control is of benefit to investigate, to decipher if the results are driven by the smaller sized NPs or another attribute. Also, it is relevant that perhaps the toxicity of smaller WO3 NPs is a consequence of their greater capacity to release W ions, which mediate the observed toxicity, but this is uncertain at this time. However, it has been repeatedly demonstrated that NPs toxicity is driven by their size and dose, so that when administered at an equal-mass dose, smaller particles have a larger surface area, and therefore exhibit a greater toxic potential. Further, the information available on the effect of NPs on the environment and human occupational exposure are limited, satisfactory risk assessment of the safeness of NPs cannot be made [52,59].

5. Conclusion

To generate reliable toxicological data for WO_3 NPs and MPs, not only different primary particle size, zeta potential, crystal structure and surface area but also validated standard protocols (genotoxicity, biochemical, histopathology and biodistribution) were employed in this study. From our results, WO_3 NPs were found to have a little toxic effect at the highest dose through the 28 days repeated oral exposure under the OECD test guideline 407 (2008). However, WO_3 MPs did not incite any toxicity attributes in comparison to controls. High doses were intentionally used in this study to obtain detectable quantities after accumulation into the rats and were not for representing exposure to humans. In the current investigation, the observed toxicity may be due to oxidative stress and inflammation though the exact mechanism is not fully understood. Although, there is a scope that other toxicity pathways may be involved. The positive results of genotoxic effects at only high doses signify that WO_3 NPs have a low efficiency in inducing genotoxicity. However, there may be a possibility that various exposure routes or long term exposure can cause genotoxicity in different kinds of organs. Hence, there is always a wide scope for more rigorous toxicity study to get a firm conclusion. Nevertheless, the findings of the present study will add to the increasing knowledge of WO_3 NPs toxicity.

Conflict of interest statement

There is no conflict of interest related to this research.

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References

[1] S.J. Bradfield, P. Kumar, J.C. White, S.D. Ebbs, Zinc, copper, or cerium accumulation from metal oxide nanoparticles or ions in sweet potato: yield effects and projected dietary intake from consumption, Plant Physiol. Biochem. (2016).
[2] M.E. Vance, T. Kuiken, E.P. Vejerano, S.P. McGinnis, M.F. Hochella Jr., D. Rejeski, M.S. Hull, Nanotechnology in the real world: redeveloping the nanomaterial consumer products inventory, Beilstein J. Nanotechnol. 6 (2015) 1769–1780.
[3] A.A. Keller, A. Lazareva, Predicted releases of engineered nanomaterials: from global to regional to local, Environ. Sci. Technol. Lett. 1 (2013) 65–70.
[4] D. McShan, P.C. Ray, H. Yu, Molecular toxicity mechanism of nanosilver, J. Food Drug Anal. 22 (2014) 116–127.
[5] A. Nel, T. Xia, L. Mädler, N. Li, Toxic potential of materials at the nanolevel, Science 311 (2006) 622–627.
[6] S. Arora, J.M. Rajwade, K.M. Paknikar, Nanotoxicology and in vitro studies: the need of the hour, Toxicol. Appl. Pharmacol. 258 (2012) 151–165.
[7] K. Tomankova, J. Horakova, M. Harvanova, L. Malina, J. Soukupova, S. Hradilova, K. Kejlova, J. Malohlava, L. Licman, M. Dvorakova, Cytotoxicity, cell uptake and microscopic analysis of titanium dioxide and silver nanoparticles in vitro, Food Chem. Toxicol. 82 (2015) 106–115.
[8] I. Grissa, J. Elghoul, L. Ezzi, S. Chakroun, E. Kerkeni, M. Hassine, L. El Mir, M. Mehdi, H.B. Cheikh, Z. Haouas, Anemia and genotoxicity induced by sub-chronic intragastric treatment of rats with titanium dioxide nanoparticles, Mut. Res. Genet. Toxicol. Environ. Mutagen. 794 (2015) 25–31.
[9] Z. Máté, E. Horváth, G. Kozma, T. Simon, Z. Kónya, E. Paulik, A. Papp, A. Szabó, Size-dependent toxicity differences of intratracheally instilled manganese oxide nanoparticles: conclusions of a subacute animal experiment, Biol. Trace Elem. Res. 171 (2016) 156–166.
[10] H.K. Lindberg, G.C.-M. Falck, J. Catalán, A.J. Koivisto, S. Suhonen, H. Järventaus, E.M. Rossi, H. Nykäsenoja, Y. Peltonen, C. Moreno, Genotoxicity of inhaled nanosized TiO 2 in mice, Mut. Res. Genet. Toxicol. Environ. Mutagen. 745 (2012) 58–64.
[11] R. Abbasalipourkabir, H. Moradi, S. Zarei, S. Asadi, A. Salehzadeh, A. Ghafourikhosroshahi, M. Mortazavi, N. Ziamajidi, Toxicity of zinc oxide nanoparticles on adult male Wistar rats, Food Chem. Toxicol. 84 (2015) 154–160.
[12] Y.S. Kim, J.S. Kim, H.S. Cho, D.S. Rha, J.M. Kim, J.D. Park, B.S. Choi, R. Lim, H.K. Chang, Y.H. Chung, Twenty-eight-day oral toxicity, genotoxicity, and gender-related tissue distribution of silver nanoparticles in Sprague-Dawley rats, Inhal. Toxicol. 20 (2008) 575–583.
[13] J. Wang, M.F. Rahman, H.M. Duhart, G.D. Newport, T.A. Patterson, R.C. Murdock, S.M. Hussain, J.J. Schlager, S.F. Ali, Expression changes of dopaminergic system-related genes in PC12 cells induced by manganese silver, or copper nanoparticles, Neurotoxicology 30 (2009) 926–933.
[14] Y.-H. Lee, F.-Y. Cheng, H.-W. Chiu, J.-C. Tsai, C.-Y. Fang, C.-W. Chen, Y.-J. Wang, Cytotoxicity oxidative stress, apoptosis and the autophagic effects of silver nanoparticles in mouse embryonic fibroblasts, Biomaterials 35 (2014) 4706–4715.
[15] M. Kumari, S.I. Kumari, S.S.K. Kamal, P. Grover, Genotoxicity assessment of cerium oxide nanoparticles in female Wistar rats after acute oral exposure, Mut. Res. Genet. Toxicol. Environ. Mutagen. 775 (2014) 7–19.
[16] P.B. Tchounwou, C.G. Yedjou, A.K. Patlolla, D.J. Sutton, Heavy metal toxicity and the environment, Mol. Clin. Environ. Toxicol. Springer (2012) 133–164.
[17] G. Zhou, Y. Hou, L. Liu, H. Liu, C. Liu, J. Liu, H. Qiao, W. Liu, Y. Fan, S. Shen, Preparation and characterization of NiW?nHA composite catalyst for hydrocracking, Nanoscale 4 (2012) 7698–7703.
[18] Y.-D. Huh, J.-H. Shim, Y. Kim, Y.R. Do, Optical properties of three-band white light emitting diodes, J. Electrochem. Soc. 150 (2003) H57–H60.
[19] X. Li, G. Zhang, F. Cheng, B. Guo, J. Chen, Synthesis, characterization, and gas-sensor application of WO3 nanocuboids, J. Electrochem. Soc. 153 (2006) H133–H137.
[20] K. Huang, Q. Pan, F. Yang, S. Ni, D. He, Synthesis and field-emission properties of the tungsten oxide nanowire arrays, Physica E: Low-dimensional, Syst. Nanostruct. 39 (2007) 219–222.
[21] E. Llobet, G. Molas, P. Molinas, J. Calderer, X. Vilanova, J. Brezmes, J. Sueiras, X. Correig, Fabrication of highly selective tungsten oxide ammonia sensors, J. Electrochem. Soc. 147 (2000) 776–779.
[22] C.S. Rout, A. Govindaraj, C. Rao, High-sensitivity hydrocarbon sensors based on tungsten oxide nanowires, J. Mater. Chem. 16 (2006) 3936–3941.
[23] A. Ashkarran, M. Ahadian, S.M. Ardakani, Synthesis and photocatalytic activity of WO3 nanoparticles prepared by the arc discharge method in deionized water, Nanotechnology 19 (2008) 195709.
[24] T. Ryu, H.Y. Sohn, K.S. Hwang, Z.Z. Fang, Chemical vapor synthesis (CVS) of tungsten nanopowder in a thermal plasma reactor, Int. J. Refract. Met. Hard Mater. 27 (2009) 149–154.
[25] M.A. Syed, U. Manzoor, I. Shah, S.H.A. Bukhari, Antibacterial effects of Tungsten nanoparticles on the Escherichia coli strains isolated from catheterized urinary tract infection (UTI) cases and Staphylococcus aureus, New Microbiol. 33 (2010) 329.
[26] G. Hasegawa, M. Shimonaka, Y. Ishihara, Differential genotoxicity of chemical properties and particle size of rare metal and metal oxide nanoparticles, J. Appl. Toxicol. 32 (2012) 72–80.
[27] H. Turkez, E. Sonmez, O. Turkez, Y.I. Mokhtar, A.D. Stefano, G. Turgut, The risk evaluation of tungsten oxide nanoparticles in cultured rat liver cells for its safe applications in nanotechnology, Braz. Arch. Biol. Technol. 57 (2014) 532–541.
[28] A. Ivask, T. Titma, M. Visnapuu, H. Vija, A. Kakinen, M. Sihtmae, S. Pokhrel, L. Madler, M. Heinlaan, V. Kisand, Toxicity of 11 metal oxide nanoparticles to three mammalian cell types in vitro, Curr. Top. Med. Chem. 15 (2015) 1914–1929.
[29] H. Turkez, B. Cakmak, K. Celik, Evaluation of the potential in vivo genotoxicity of tungsten (VI) oxide nanopowder for human health, Key Eng. Mater. Trans. Tech. Publ (2013) 89–92.
[30] M.V. Prajapati, O.O. Adebolu, B.M. Morrow, J.M. Cerreta, Evaluation of pulmonary response to inhaled tungsten (IV) oxide nanoparticles in golden Syrian hamsters, Exp. Biol. Med. (2016) (1535370216665173).
[31] R. Tice, E. Agurell, D. Anderson, B. Burlinson, A. Hartmann, H. Kobayashi, Y. Miyamae, E. Rojas, J. Ryu, Y. Sasaki, Single cell gel/comet assay: guidelines for in vitro and in vivo genetic toxicology testing, Environ. Mol. Mutagen. 35 (2000) 206–221.
[32] OECD, Test No. 489: In Vivo Mammalian Alkaline Comet Assay, OECD Guidelines for the Testing of Chemicals , Section 4, OECD Publishing, Paris, 2016.
[33] Y. Miyamae, M. Yamamoto, Y.F. Sasaki, H. Kobayashi, M. Igarashi-Soga, K. Shimoi, M. Hayashi, Evaluation of a tissue homogenization technique that isolates nuclei for the in vivo single cell gel electrophoresis (comet) assay: a collaborative study by five laboratories, Mut. Res. Genet. Toxicol. Environ. Mutagen. 418 (1998) 131–140.
[34] B.L. Pool-Zobel, N. Lotzmann, M. Knoll, F. Kuchenmeister, R. Lambertz, U. Leucht, H.G. Schröder, P. Schmezer, Detection of genotoxic effects in human gastric and nasal mucosa cells isolated from biopsy samples, Environ. Mol. Mutagen. 24 (1994) 23–45.
[35] S.P. Singh, S. Chinde, S.S.K. Kamal, M. Rahman, M. Mahboob, P. Grover, Genotoxic effects of chromium oxide nanoparticles and microparticles in Wistar rats after 28 days of repeated oral exposure, Environ. Sci. Pollut. Res. 23 (2016) 3914–3924.
[36] H. Yatzidis, Measurement of transaminases in serum, Nature 186 (1960) 79–80.
[37] D. Jollow, J. Mitchell, J. Gillette, J. Gillette, Bromobenzene-induced liver necrosis. Protective role of glutathione and evidence for 3, 4-bromobenzene oxide as the hepatotoxic metabolite, Pharmacology 11 (1974) 151–169.
[38] A.K. Sinha, Colorimetric assay of catalase, Anal. Biochem. 47 (1972) 389–394.
[39] H. Ohkawa, N. Ohishi, K. Yagi, Assay for lipid peroxides in animal tissues by thiobarbituric acid reaction, Anal. Biochem. 95 (1979) 351–358.
[40] M. Gómez, D.J. Sánchez, J.M. Llobet, J. Corbella, J. Domingo, The effect of age on aluminum retention in rats, Toxicology 116 (1997) 1–8.
[41] J. Wang, G. Zhou, C. Chen, H. Yu, T. Wang, Y. Ma, G. Jia, Y. Gao, B. Li, J. Sun, Acute toxicity and biodistribution of different sized titanium dioxide particles in mice after oral administration, Toxicol. Lett. 168 (2007) 176–185.
[42] H.L. Karlsson, J. Gustafsson, P. Cronholm, L. Möller, Size-dependent toxicity of metal oxide particles-a comparison between nano-and micrometer size, Toxicol. Lett. 188 (2009) 112–118.
[43] M. Arnold, A. Badireddy, M. Wiesner, R. Di Giulio, J. Meyer, Cerium oxide nanoparticles are more toxic than equimolar bulk cerium oxide in Caenorhabditis elegans, Arch. Environ. Contam. Toxicol. 65 (2013) 224–233.
[44] M. Kumari, S.I. Kumari, P. Grover, Genotoxicity analysis of cerium oxide micro and nanoparticles in Wistar rats after 28 days of repeated oral administration, Mutagenesis 29 (2014) 467–479.
[45] S.P. Singh, M. Kumari, S.I. Kumari, M.F. Rahman, S.K. Kamal, M. Mahboob, P. Grover, Genotoxicity of nano-and micron-sized manganese oxide in rats after acute oral treatment, Mut. Res. Genet. Toxicol. Environ. Mutagen. 754 (2013) 39–50.
[46] V. Sharma, P. Singh, A.K. Pandey, A. Dhawan, Induction of oxidative stress, DNA damage and apoptosis in mouse liver after sub-acute oral exposure to zinc oxide nanoparticles, Mut. Res. Genet. Toxicol. Environ. Mutagen. 745 (2012) 84–91.
[47] L.P. Sycheva, V.S. Zhurkov, V.V. Iurchenko, N.O. Daugel-Dauge, M.A. Kovalenko, E.K. Krivtsova, A.D. Durnev, Investigation of genotoxic and cytotoxic effects of micro-and nanosized titanium dioxide in six organs of mice in vivo, Mut. Res. Genet. Toxicol. Environ. Mutagen. 726 (2011) 8–14.
[48] Y. Duan, J. Liu, L. Ma, N. Li, H. Liu, J. Wang, L. Zheng, C. Liu, X. Wang, X. Zhao, Toxicological characteristics of nanoparticulate anatase titanium dioxide in mice, Biomaterials 31 (2010) 894–899.
[49] S. Gui, Z. Zhang, L. Zheng, Y. Cui, X. Liu, N. Li, X. Sang, Q. Sun, G. Gao, Z. Cheng, Molecular mechanism of kidney injury of mice caused by exposure to titanium dioxide nanoparticles, J. Hazard. Mater. 195 (2011) 365–370.
[50] M. Kumari, S. Rajak, S.P. Singh, S.I. Kumari, P.U. Kumar, U.S. Murty, M. Mahboob, P. Grover, M.F. Rahman, Repeated oral dose toxicity of iron oxide nanoparticles: biochemical and histopathological alterations in different tissues of rats, J. Nanosci. Nanotechnol. 12 (2012) 2149–2159.

[51] U.A. Reddy, P. Prabhakar, M. Mahboob, Biomarkers of oxidative stress for in vivo assessment of toxicological effects of iron oxide nanoparticles, Saudi J. Biol. Sci. (2015).
[52] I.C. Lee, J.W. Ko, S.H. Park, N.R. Shin, I.S. Shin, C. Moon, J.H. Kim, H.C. Kim, J.C. Kim, Comparative toxicity and biodistribution assessments in rats following subchronic oral exposure to copper nanoparticles and microparticles, Part. Fibre Toxicol. 13 (2016) 56.
[53] H. Liu, D. Yang, H. Yang, H. Zhang, W. Zhang, Y. Fang, Z. Lin, L. Tian, B. Lin, J. Yan, Comparative study of respiratory tract immune toxicity induced by three sterilisation nanoparticles: silver, zinc oxide and titanium dioxide, J. Hazard. Mater. 248 (2013) 478–486.
[54] S.W. Wijnhoven, W.J. Peijnenburg, C.A. Herberts, W.I. Hagens, A.G. Oomen, E.H. Heugens, B. Roszek, J. Bisschops, I. Gosens, D. Van De Meent, Nano-silver—a review of available data and knowledge gaps in human and environmental risk assessment, Nanotoxicology 3 (2009) 109–138.
[55] A. Patri, T. Umbreit, J. Zheng, K. Nagashima, P. Goering, S. Francke-Carroll, E. Gordon, J. Weaver, T. Miller, N. Sadrieh, Energy dispersive X-ray analysis of titanium dioxide nanoparticle distribution after intravenous and subcutaneous injection in mice, J. Appl. Toxicol. 29 (2009) 662–672.
[56] K. Shapero, F. Fenaroli, I. Lynch, D.C. Cottell, A. Salvati, K.A. Dawson, Time and space resolved uptake study of silica nanoparticles by human cells, Mol. Biosyst. 7 (2011) 371–378.
[57] G. Sonavane, K. Tomoda, K. Makino, Biodistribution of colloidal gold nanoparticles after intravenous administration: effect of particle size, Colloids Surf. B: Biointerfaces 66 (2008) 274–280.
[58] H.J. Johnston, G. Hutchison, F.M. Christensen, S. Peters, S. Hankin, V. Stone, A review of the in vivo and in vitro toxicity of silver and gold particulates: particle attributes and biological mechanisms responsible for the observed toxicity, Crit. Rev. Toxicol. 40 (2010) 328–346.
[59] F. Gottschalk, T. Sun, B. Nowack, Environmental concentrations of engineered nanomaterials: review of modeling and analytical studies, Environ. Pollut. 181 (2013) 287–300.