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Dr. Paramjit Grover

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Indian Institute of Chemical Technology
Biology Division
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India

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Principal Investigator:
Dr.Ms.Paramjit Grover, Senior Principal Scientist, Biology Division,
Indian Institute of Chemical Technology, Hyderabad, India
Tel: +91-40-27193135, E.mail:paramgrover@gmail.com, grover@iict.res.in
Co-Investigators:
Dr.M.F.Rahman, Senior Technical Officer,
Indian Institute of Chemical Technology, Hyderabad, India

Dr.M.Mahboob, Senior Technical Officer,
Indian Institute of Chemical Technology, Hyderabad, India
Project Co-coordinator:
Dr.USN Murty, Chief Scientist and Head Biology Division,
Indian Institute of Chemical Technology, Hyderabad, India
Tel: +91-40-27193134, E.mail:murty_usn@yahoo.com

AFOSR/AOARD Program Manager: Lt Col Kristopher Ahlers

Collaborators: Dr. Saber Hussain, Senior Scientist, AFRL, WPAFB, OH

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Abstract

The study in this project was undertaken to document the effect of 24 hours exposure of cerium oxide (CeO_2), cobalt oxide (Co_3O_4) and tungsten oxide (WO_3) nanoparticles (NPs) on different cell lines. The study consisted of physicochemical characterization, cytotoxic, genotoxic and oxidative stress responses due to *in vitro* exposure to different doses of NPs and their microparticles (MPs). The physicochemical properties like size, size distribution, state of dispersion and zeta potential of these NPs are the important factors to study their toxicity. We developed a novel *in vitro* system to systematically assess the interaction of the NMs and their bulk particles within the cell. Bulk analogues were used to find out the size effect on the toxicity. Cytotoxicity was evaluated by 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide Assay (MTT) and lactate dehydrogenase (LDH) assays, whereas genotoxicity was assessed using the cytokinesis-block micronucleus and comet assays. A battery of assays including lipid peroxidation (LPO), reactive oxygen species (ROS), hydrogen peroxide (H_2O_2), reduced glutathione (GSH), nitric oxide (NO), glutathione reductase (GR), glutathione peroxidase, superoxide dismutase (SOD), catalase (CAT) and glutathione S-transferase were performed to test the hypothesis that ROS was responsible for the toxicity of the NPs. The MTT cytotoxicity assay was performed in three cell lines viz., human embryonic kidney cell line (HEK 293) and human neuroblastoma cell line (IMR 32). The mean size of CeO_2, Co_3O_4, and WO_3 NMs was 25, 16 and 52 nm respectively. The DLS data revealed the aggregation of CeO_2, Co_3O_4, and WO_3 NPs in suspension. Zeta potential of CeO_2, Co_3O_4, and WO_3 NPs in DMEM was determined by LDV and found to be -7.74, -8.20, and -6.03 respectively. In the culture medium NPs showed a slight increase in the size with a concomitant decrease in zeta-potential. The MTT cytotoxicity assay with three cell lines exhibited dose dependent loss in the viability with CeO_2, Co_3O_4, and WO_3 NPs. There were variations in the sensitivity of cells to the different NMs. The IMR32 cell lines exposed to CeO_2 NPs for 24 hours exhibited IC_{50} >300 µg/ml. Similarly, HEK293 cell lines exposed to Co_3O_4 NPs and WO_3 NPs showed IC_{50} >300 µg/ml. Bulk compounds of these three metal oxides were less cytotoxic than their NMs with all the three cell lines tested and the IC_{50} observed was > 300 µg/ml. To investigate the potential role of oxidative stress as a mechanism of toxicity, reactive oxygen species (ROS), nitric oxide (NO), lactate dehydrogenase (LDH) level and reduction in the total glutathione (GSH) were estimated. The results showed that, there was significant induction of ROS, NO, LDH and GSH level

observed in IMR32 cells treated with CeO_2 NPs from 100 µg/ml onwards, whereas in their bulk no significant changes were observed. Similarly, HEK293 cells treated with Co_3O_4 NPs showed significant increase in ROS, membrane leakage of LDH and NO and reduction in the total GSH at the dose level of 50 µg/ml onwards however, bulk were not found to induce any significant changes. ROS production was significantly increased from 30µg/ml ($p<0.05$) of Co_3O_4 NPs, whereas its bulk 40µg/ml ($p<0.05$) compared to control. Moreover, cell viabilities at different concentrations of WO_3 NPs were decreased in a dose dependent manner. Further, WO_3 NPs significantly increased the NO levels in concentration dependent manner from 50µg/ml. A significant dose dependent increase in micronuclei (MN) frequency was induced by CeO_2 NPs with IMR32 cells and by $Co3O_4$ NP in HEK293 cell cultures. WO_3 NPs showed an increase in MN frequency in comparison to the bulk material only. Comet assay of the NMs and bulk treated cells showed a concentration dependent increase in the percentage of tail DNA, however only the NMs showed a significant increase.

1. Introduction

In view of the fast-growing industrial applications of engineered nanomaterials (NMs), the evaluation of their toxicity potential and of their mode of action is a necessity to obtain adequate hazard/risk assessment and to produce safer and sustainable NMs.The prefix "nano" was specifically coined for particles containing tens or hundreds of atoms, with dimensions at the scale of less than 100 nm **(Buzea et al., 2007)**. It is this small size which is fundamental to the field of nanotechnology, although other physicochemical properties influence the physical, biological and toxicological properties of these manufactured materials **(Edwards-Jones, 2009)**. The principal reason for producing and exploiting NMs is that their behaviour is fundamentally different from the bulk form of the same compound **(Edwards-Jones, 2009)**. Nanotechnologies are the design, characterization, production and application of structures, devices and systems by controlling shape and size at the nanoscale level. It is a rapidly advancing discipline with a wide range of applications, including those in medicine and industry. Nanoparticles (NPs) have a specific capacity for drug loading, high superparamagnetism, efficient photoluminescence, in the targeted delivery of imaging agents and anti-cancer drugs. The overwhelming applications of NMs due to their superior physicochemical features bestow enormous potential for human exposure and environmental release. Therefore, using NMs without fully understanding their potential health risks would be dangerous **(Nel et al., 2006)**. The current lack of knowledge in this regard has led to an urgent call for the establishment of principles and test procedures to ensure the safe manufacture and use of NMs in the marketplace. An *in vitro* assessment of panel of engineered NMs using a different cell line assessing cytotoxicity, pro-inflammatory response, and oxidative stress may be an important approach in order to understand the possible toxicity and their mechanisms of toxicity. Oxidative stress has been implicated as a possible mechanism for NMs toxicity, hence effects on the human renal proximal tubule epithelial cells (HK-2) treated with a panel of engineered NMs were evaluated **(Kermanizadeh et al., 2013)**. In the current investigation, the toxicological interaction of cerium oxide (CeO_2) was studied with IMR32 cells whereas cobalt oxide (Co_3O_4) and tungsten oxide (WO_3) NMs with and HEK293 cell lines. CeO_2 NMs are amongst the most widely used rare earth compounds finding applications in industrial and commercial products. The industrial applications includes its uses as a polishing agents, ultraviolet absorbing compound in sunscreen, solid

electrolytes in solid oxide fuel cells, as a fuel additive to promote combustion and in automotive exhaust catalyst **(Shah et al., 2012)**.

Co_3O_4 NMs applications are in information storage, magnetic fluid, catalysts, pigments, coatings, catalysis, sensors, anode materials in rechargeable batteries, solar energy absorbers etc. WO_3 NMs are of great interest due to their potential use as electrochromic, gas sensing and photo catalyst materials. Therefore, there is urgent requirement for the toxicity study of these NMs at the cellular level in order to understand and conclude the real site of action of these NMs.There are a number of toxicological endpoints that researchers have used to assess the potential adverse effects that NMs may have on organs of the human body. These include numerous biochemical- and molecular-based testing strategies, investigating the potential for NMs to cause cytotoxicity, inflammation, oxidative stress, cell proliferation and genotoxicity. As the physical and chemical properties of NPs can vary significantly from those of their bulk counterparts, microparticles (MPs) were used to compare the size effect. The characterization of NPs before commencing the study is fundamental for any toxicity study. Therefore, in this research we performed transmission electron microscope (TEM), Dynamic light scattering (DLS) and laser Doppler velocimetry (LDV) studies to know the size, mean hydrodynamic diameter and zeta potential respectively of CeO_2 NPs, Co_3O_4 and WO_3. The use of DLS in characterization of NPs is considered indispensible as it measures the size in the exposure medium to give a true idea of particle distribution. In this study cytotoxicity was evaluated by the formazan reduction 3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyl tetrazolium bromide (MTT) assay. It is a viability test that works efficiently in the vast majority of cases, so that it is often used for evaluating in vitro cytotoxicity of broad range of NPs **(Bucchianico et al., 2013; Sharma et al., 2009)**. Further, this part of research is intended to get the details regarding the possible mechanism of oxidative stress involved in the alteration caused by NMs and their interaction within cells. Hence, in order to conceptualize the mechanism of NPs toxicity *in vitro*, it has been suggested that lipid peroxidation (LPO), glutathione (GSH), lactate dehydrogenase (LDH) levels and nitric oxide (NO) along with battery of marker enzymes of antioxidative defense system play an important role in NP elicited DNA damage, cell membrane disruption and subsequent cell death **(Lee et al., 2012; Sharma et al., 2009)**. Therefore, in the present study LPO, ROS, hydrogen peroxide (H_2O_2), GSH, LDH, NO, glutathione reductase (GR), glutathione peroxidase (GPx), superoxide dismutase (SOD), catalase (CAT) and glutathione S-transferase (GST) estimations were carried out.

The importance of a genotoxicological approaches during the assessment of toxicity of NPs has been highlighted by various researchers. The *in vitro* micronucleus (MN) test has become an attractive and one of the standard cytogenetic tests for genotoxicity testing. It is a multi-target genotoxic endpoint, which is very helpful in the assessment of clastogenic and aneugenic events and also some epigenetic effects, which is simple to score, accurate and applicable in different cell types **(Kirsch-Volders et al., 2011)**. The cytokinesis-block micronucleus (CBMN) assay is the preferred method for measuring micronuclei (MNi) in cultured cell lines because scoring is specifically restricted to once divide bi-nucleated cells **(Fenech, 2006)**. At the same time CBMN assay is good approach to evaluate other damage events like nucleoplasmic bridges, a biomarker of DNA misrepair and/or telomere end-fusions and nuclear buds (NBUDs), a biomarker of elimination of amplified DNA and/or DNA repair complexes and the number of apoptotic and necrotic cells **(Fenech, 2007)**. The single cell gel electrophoresis (SCGE) or comet assay is important to assess the DNA damaging potential of these particles. It is a promising genotoxicity assay *in vitro* and *in vivo* because of rapid and sensitive detection of DNA damage in individual cells and thus, it has been used in a broad spectrum of genetic studies **(Olive and Banath, 2006)**. Hence, the CBMN and comet assays were additionally performed in the present study.

2. Experiment

2.1. Chemicals:

Cerium oxide bulk (CeO_2 , 99.9%, <5µm, CAS No.1306-38-3) ,Cerium oxide nanopowder (CeO_2 <25nm, CAS No.1306-38-3), Cobalt oxide nanopowder (Co_3O_4 99.8%, <50nm CAS No. 138-06-1), Cobalt oxide bulk (Co_3O_4 99.8%, <10µm CAS No. 138-06-1), Tungsten oxide nanopowder (WO_3 99.8%, <100nm CAS No. 1314-35-8), Tungsten oxide bulk (WO_3 99%, <20µm CAS No. 1314-35-8) were purchased from Sigma Chemical Co. Ltd. (St. Louis, MO, USA). Phosphate buffered saline (Ca_2+, Mg_2+ free; PBS), Dulbecco's modified eagle medium (DMEM), trypsin–EDTA, fetal bovine serum (FBS), antibiotic solution (10,000 U/ml penicillin,10 mg/ml streptomycin) were also purchased from Sigma Chemical Co. Ltd. (St. Louis, MO, USA). All other chemicals were obtained locally and were of analytical reagent grade. Cell culture plastic wares were obtained from Tarsons Products Pvt.Ltd. (Kolkata, India).

2.2. Cell culture:

- Human embryonic kidney cell line (HEK 293) was grown in DMEM supplemented with 10% FBS, 0.2% sodium bicarbonate and 10 ml/L antibiotic solution at 37^{o}C under a humidified atmosphere of 5% CO_2/95% air.
- Human neuroblastoma cell line (IMR 32) was obtained from American Type Culture Collection (ATCC) and cultured in DMEM supplemented with 10% FBS, 0.2% sodium bicarbonate and 10 ml/L antibiotic solution at 37^{o}C under a humidified atmosphere of 5% CO_2/95% air.

2.3. Characterization

Transmission Electron Microscopy:

Transmission Electron Microscope (TEM) characterization was performed to obtain nanoparticle size and morphology on a TEM (JEOL, Japan) at an accelerating voltage of 120 kV. CeO_2, Co_3O_4 and WO_3 NPs were examined after suspension in Milli-Q water and subsequent deposition onto TEM grids. Information on mean size and standard error was calculated by measuring over 100 NPs in random fields of view, in addition to images that show general morphology of the NPs.

Dynamic light scattering (DLS) and Laser Doppler velocimetry (LDV):

Dynamic light scattering (DLS) and zeta potential measurements were performed with a Zetasizer Nano ZS (Malvern Instruments), provided with a He/Ne laser of 633 nm wavelength. DLS and Laser Doppler velocimetry (LDV) were used for the size and charge characterization of CeO_2, Co_3O_4 and WO_3 NPs in solution, after suspension in DMEM with ultrasonication. The freshly prepared stock solutions were ultrasonicated using a probe sonicator for 10 min. Samples thus prepared were transferred to a 1.5 ml square cuvette for DLS measurements and 1 ml was transferred to a Malvern Clear Zeta Potential cell for LDV measurement. Average size was calculated by the software from the intensity, volume and number distributions measured.

Dispersion of test materials

Metal oxide NPs (CeO_2, Co_3O_4 and WO_3) and their bulk were dispersed in PBS. Homogenous dispersion was obtained by physical mixing and sonication for 5-10 minutes.

Different stock solutions of metal oxide NPs were obtained by diluting with DMEM with 5% FBS to obtain final concentration of 10, 20, 30, 40, 50, 100, 150, 200, 250 and 300µg/ml. 10µl of nanoparticles suspension was added to 100µl of exposure media in 96 well plate. Constant mixing was done before exposure to prevent the NPsfrom settling down in the solution.

2.4. Cell viability 3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyl Tetrazolium Bromide Assay (MTT Cytotoxicity Assay)

Metal oxide NPs (CeO_2, Co_3O_4 and WO_3) cytotoxicity was assessed using MTT assay following the method described by **Hansen et al., (1989)** for IMR32 and HEK 293 cells respectively were used. The assay is dependent on the reduction of the tetrazolium salt MTT (3-(4,5-dimethylthazol-2-yl)-2,5-diphenyl tetrazolium bromide) by the mitochondrial dehydrogenase of viable cells to form a blue formazan product dissolved in DMSO and read at 570 nm. Briefly, 100µl of mammalian cell lines were suspended in 96 wells plate after 50-60% confluency cells were treated to different concentrations of NMs suspended in DMEM media with 5% serum for a time period of 24hr. Then, medium in each well was discarded and fresh supplemented medium (100 µl) followed by 10 µl of MTT solution (5 mg/ml in PBS, filtered sterile) was added. Medium blank was put up with only medium (100 µl) and MTT (10 µl). Plates were incubated at 37 °C for 2 h. The formazan crystals formed by the action of mitochondrial dehydrogenase on MTT was dissolved in 100ul of DMSO, Absorbance was measured at 570 nm using Spectra Max plus 384 UV-Visible plate reader.

Dispersion of test materials

Metal oxide NPs (CeO_2, Co_3O_4 and WO_3) and their bulk were dispersed in PBS. Homogenous dispersion was obtained by physical mixing and sonication for 5-10 minutes. Different stock solutions of metal oxide NPs were obtained by diluting with DMEM with 5% FBS to obtain final concentration of 10, 20, 30, 40, 50, 100, 150, 200, 250 and 300µg/ml. 10 µl of nanoparticles suspension was added to 100 µl of exposure media in 96 well plate. Constant mixing was done before exposure to prevent the NPs from settling down in the solution.

2.5. Lactate dehydrogenase release

LDH release was measured in 96-well plate with 60-80 % confluent cells treated with different concentrations of NPs and MPs. This estimation was done according to the procedures described in Cytoscan^{TM} LDH cytotoxicity assay kit (Geno Biosciences Pvt. Ltd.) and LDH release was measured spectrophtometrically at 340 nm using Spectra Max Plus 384 UV-Visible plate reader (Molecular Devices, Sunnyvale, CA, USA). The percent of LDH activity was calculated by dividing the amount of activity in the medium by the total activity (medium and cell lysate).

2.6. Measurement of reactive oxygen species

Intracellular superoxide was estimated fluorometrically using the oxidation-sensitive fluorescent probe DCF-DA **(Royall and Ischiropoulos, 1993)**. In the 96-well plate containing 60-80% confluent cells, DCF-DA (20 μM) was added to each well and incubated for 30 minutes. The cells were washed with PBS to remove extra DCF-DA and then 5% culture medium was added. Further, cells were inoculated with different concentrations of NPs and MPs and incubated for 24 h and finally PBS was added to each well and fluorescence intensity was read on a spectrofluorometer (Dynex Technologies, VA, USA) at the excitation and emission wavelengths of 485 and 528 nm respectively.

2.7. Hydrogen peroxide assay

The intracellular production of H_2O_2 was measured with DHR, as described by **Park et al. (2008b)**. During the cellular production of ROS, the non-fluorescent DHR was oxidized by H_2O_2 and irreversibly converted to the green fluorescent compound rhodamine 123 (R123). R123 is membrane-impermeable and accumulates in the cells. An aliquot of DHR (to produce a concentration of 10 μM in each well) was added to each 96-well plate and pre-incubated for 30 min at 37 °C. Thereafter, the medium was removed and cells were incubated with NPs and MPs for 24 h at 37 °C. After incubation, fluorescence intensities of each well were analyzed by spectrofluorometer with excitation filter 485 nm and emission filter 535 nm.

2.8. Nitric oxide assay

NO concentration was determined via the Griess reaction. Briefly, cells were plated in 96-well plate up to 60-80% confluency and inoculated with different concentrations NPs and MPs for 24 h. An aliquot of 100 µl from each well were mixed with same amount of Griess reagent in wells of a separate 96-well plate. After 15 min incubation at room temperature, the developed pink colour was read at 540 nm **(Lee et al., 2012)**.

2.9. Preparation of cell lysate and protein estimation

The cells were allowed to grow up to 60-80% confluency before treatment with different concentrations of CeO_2, Co_3O_4 and WO_3 NPs and their MPs in 12-well plate for 24 h. Thereafter, cells were washed with PBS, and 100 µl lysis buffer (146 mM NaCl, 0.62 mM EDTA, 5% Triton X-100 and 10 mM Tris buffer) was added to each well and left for 30 min on ice. The cell lysate were centrifuged at 13,000 rpm at 4°C for 5 min. The supernatant was collected for further analysis. The total protein concentration was estimated in cell supernatant following the method of **Bradford (1976)**. The Bradford reagent (200 µl) was added to the 100 µl of cell supernatant in 96-well plate and incubated at room temperature (RT) for 30 min. The developed purple colour was measured at 595 nm. Bovine serum albumin was used as standard and experiment was performed in triplicates.

2.10. Lipid Peroxidation

MDA, the end product of lipid peroxidation was estimated according to the method of **Wills (1969)**. 100µl of cell supernatant was added to 2 ml of reaction mixture (15% TCA, 0.375%TBA) and 800µl of deionized water was added, mixed thoroughly, heated in boiling water bath for 20 min and cooled to room temperature. The color developed was extracted with 3 ml of butanol, centrifuged at 3000 rpm for 10 min. The top butanol layer was collected and read at 532 nm. The MDA level of the sample was calculated using an extinction coefficient of $1.56 \times 10^5\ M^{-1}cm^{-1}$. The levels of MDA were expressed as µmoles/ mg protein.

2.11. Reduced Glutathione content

The GSH content in cell supernatant was determined according to the method described by **Ellman (1959)**. The cell supernatant of 100 µl was mixed with 2000 µl GSH buffer (0.746 mM, pH 7.4), 500 µl Ellman reagent (4 mg/ml DTNB in 0.34 mM sodium citrate) and 400 µl milliQ water and mixed well. The mixture was incubated for 15 min at RT

and then absorbance of developed yellow colour was read at 412 nm wavelength. The reduced glutathione content in the cell samples was expressed as µmoles GSH/mg protein and calculated using extinction coefficient of 13600 $M^{-1}cm^{-1}$.

2.12. Superoxide dismutase activity

The estimation of SOD in cell supernatant was done by the method of **Marklund and Marklund (1974)**. Briefly, 3 ml of assay mixture contained 50 mM Tris–HCl buffer (pH 8.2) with 1mM DTPA, 45 µl of 10 mM pyrogallol in 10 mM HCl and 50 µl cell supernatant. The rate of inhibition of pyrogallol auto-oxidation after the addition of enzyme extract was noted. The amount of enzyme required to give 50% inhibition of pyrogallol auto-oxidation was considered as one unit of enzyme activity. The SOD activity was expressed in units/mg protein.

2.13. Catalase activity

CAT was estimated spectrophotometrically using the method of **Aebi et al. (1984)**. The assay mixture of 3 ml contained 0.063% H_2O_2 in 0.1M KPB pH 7.4 and 20 µl cell supernatant. The decrease in absorbance was then observed for 60 s at every 5 s interval at 240 nm. Activity was expressed as µmol of H_2O_2 decomposed per min per mg protein using a molar extinction coefficient of 43.6 M^{-1} cm^{-1}. The CAT activity was expressed as units/mg protein.

2.14. Glutathione reductase activity

GR activity was measured using the procedure described by **Carlberg and Mannervik (1985)**. GR assay consisted of potassium phosphate buffer (0.2M, pH 7), 2mM NADPH in 0.1% $NaHCO_3$ and oxidized glutathione (20 mM). Enzyme assay was carried out by pipetting 600 µl of potassium phosphate buffer, 250 µl Milli-Q water, 50 µl oxidized glutathione, 50 µl NADPH and 50 µl sample (cell supernatant) and the extinction of the sample was recorded at 340nm based on molar absorption coefficient of 6.22 M^{-1} cm^{-1} and the results was expressed in µmoles/min/mg protein.

2.15. Cytokinesis-block Micronucleus assay

The *in vitro* micronucleus test was conducted according to the **OECD guidelines 487** (adopted in 2007) in the culture cells. After cell cultures attained the 60-80% confluent stage

in 24-well plate, the cells were exposed to the NPs and MPs at different concentrations (10, 20, 50, 100 and 200 μg/ml) for 24 h. CP (0.20 μg/ml) was used as positive control. After treatment cells were aspirated and treated with freshly prepared DMEM medium containing cytochalasin B (3 μg/ml) for 18 h. For each concentration two cultures were prepared. The cells were then harvested with trypsin and centrifuged at 2000 rpm for 10 minutes. The cell pellets were dissolved in media and slides were prepared in triplicate for each culture. Slides were fixed with methanol and air dried, stained with 10% Geimsa in Sorensen buffer just before the evaluation with a Leica DM2500 microscope (Leica Microsystems, Wetzlar, Germany) using a ×1000 magnification. The scoring of slides followed the criteria adopted by **Fenech (2007)** for each endpoint. In brief, we evaluated 2000 binucleated cells per concentration (1000 binucleated cells per culture) to calculate the total numbers of MNi in 1000 binucleated cells. Cell proliferation was calculated by analyzing cytokinesis-block proliferation index (CBPI) which indicates the average number of cell cycles per cell during the period of exposure to cytochalasin B. Moreover, 500 cells were scored to evaluate the percentage of mono-, bi-, tri and multinucleated cells, and the CBPI was calculated as an index of cytotoxicity by comparing values in the treated and control cultures. Finally, other damage events were scored in once-divided binucleated cells per 1000 cells. The number of apoptotic and necrotic cells and mitotic figures per 500 cells were also evaluated.

CBPI = {(1× Number of mononucleated cells) + (2 × Number of binucleated cells) + (3 × Number of multinucleated cells)} / Total Number of cells

2.16. Comet assay

The method described by **Tice et al. (2000)** was used for the *in vitro* comet assay. The 24-well plate containing 60-70% confluent cells were inoculated with the same concentrations of different NPs and MPs. In a separate well, CP (0.20 μg/ml) was added as positive control. The cell pellets were taken out after 24 h of incubation and mixed with 0.37 % low-melting-point agarose (LMPA) in PBS. Pre-coating of microscope slides were done with 120 μl of 0.75 % normal-melting-point agarose (NMPA) in PBS and kept overnight at 37°C to solidify. The second coat of cells suspended in 0.37% LMPA (120 μl) was applied on pre-coated slides and kept at 4°C to dry. Again, third layer on slides was applied with 120 μl of plain 0.37% LMPA. Cover slip was put quickly for an even layer and allowed to dry at 4°C. After removing cover slip 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 1 h at 4°C. The

slides were then placed for 20 min in alkaline buffer (10 M NaOH, 200 mM Na_2 EDTA, pH > 13.0) and electrophoresed for 20 min at 25 V adjusted at 300 mA. The slides were neutralized in 0.4 M Tris buffer, pH 7.5, for 5 min, twice and in absolute methanol for 5 min, once. Coded slides were scored after staining with ethidium bromide (20 μg/ml) using a fluorescence microscope (Olympus, Japan) with a blue (488 nm) excitation filter and yellow (515 nm) emission (barrier) filter at ×400 magnification. The percentage of DNA in the comet tail in a total of 150 randomly selected cells per sample (three replicates, each with 50 cells per slide) was used as a measure of the amount of DNA damage. Quantification of DNA breakage was realized by using a Comet Image Analysis System, version Komet 5.5 (Single cell Gel Electrophoresis analysis company, Andor Technology 2005, Nottingham, UK). Results were reported as percentage of tail DNA.

2.17. Statistical analysis

The statistical significant change in all assays between treated and control groups were analyzed by one-way ANOVA. Results were expressed as mean ± standard deviation (S.D.). Multiple comparisons were performed by Dunnett test. All calculations were performed using Graph Pad Prism 4 Software package for windows. The statistical significance for all tests was set at $p<0.05$.

3. Results and Discussion

3.1. Characterization of CeO_2, Co_3O_4 and WO_3 nanoparticles:

TEM was used to characterize size and morphology of CeO_2, Co_3O_4 and WO_3 NPs. Mean size was calculated by measuring over 100 NPs in random field. The mean of CeO_2 NPs was 25 (Fig.1.1), for Co_3O_4 NPs was 16 (Fig.1.2) and for WO_3 NPs was 52 nm (Fig.1.3) respectively. The DLS data revealed the aggregation of CeO_2, Co_3O_4 and WO_3 NPs in suspension, which could be possibly due to physico-chemical interactions between the NPs. Hence, in order to create homogenous solution, constant resuspension is necessary prior to use. Zeta potential (ζ) of CeO_2, Co_3O_4 and WO_3 NPs in DMEM was determined by LDV, and found to be -7.74, -8.20 and -6.03 respectively. The size and charge of CeO_2, Co_3O_4 and WO_3 NPs in DMEM using TEM, DLS and LDV respectively are presented in Table 1.

Fig. 1.1 - TEM image of CeO_2 nanoparticles

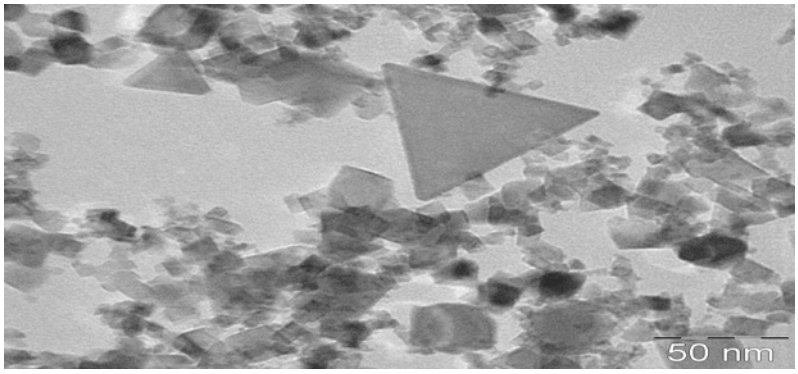


Fig.1.2 - TEM image of Co_3O_4 nanoparticles

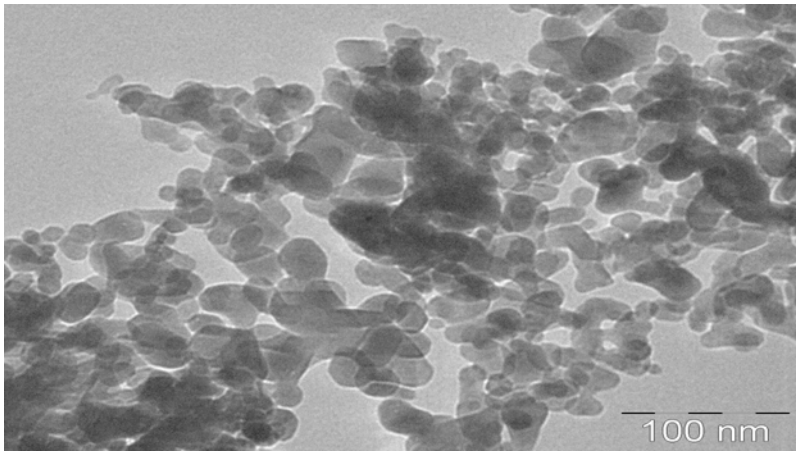


Fig. 1.3 - TEM image of WO_3 nanoparticles

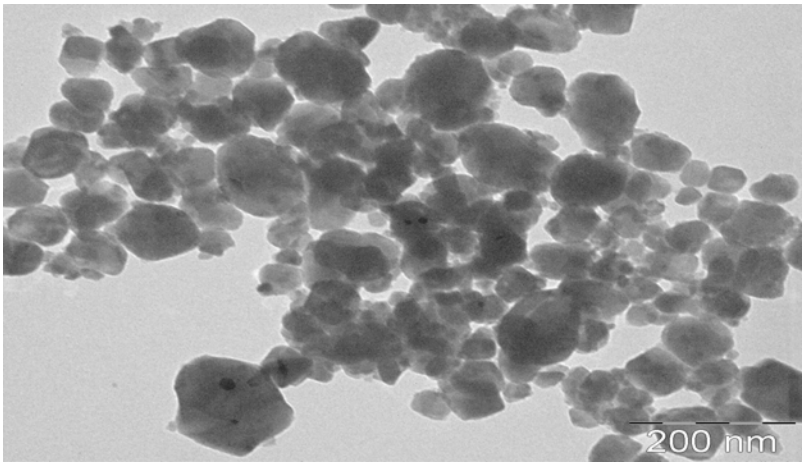


Table: 1. The size and charge of CeO_2, Co_3O_4 and WO_3 nanoparticles in DMEM.

Nano-material	Size using TEM (nm)	DLS		LDV		
		Average diameter (nm)	PDI	Zeta potential ζ (mV)	Electrophoretic Mobility(μm·cm/V/s)	pH
CeO_2	25	269.7	0.436	-7.74	-1.25	7.4
Co_3O_4	16	195.6	0.345	-8.20	-0.75	7.4
WO_3	52	203.0	0.411	-6.03	-0.75	7.4

Nanomaterials were dispersed in DMEM medium, mixing was done via probe sonication for 10min PDI=polydispersity index, DLS= dynamic light scattering, LDV= laser Doppler velocimetry, DMEM= Dulbecco's Modified Eagle's Medium

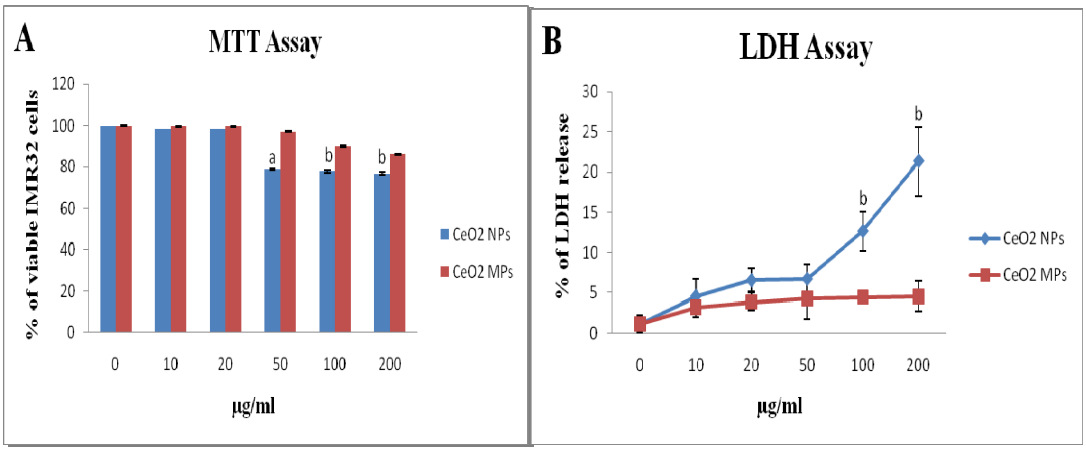
3.2. Cytotoxicity assay

A significant dose dependent decrease in mitochondrial function was observed after IMR32 cells were exposed to the CeO_2 NPs. The cell viability after 24 h incubation with CeO_2 NPs and MPs at concentrations 10, 20, 50, 100 and 200 μg/ml is shown in Figure 2A. The IC_{50} calculated for CeO_2 NP_S was 1.09 mg/ml and for CeO_2 MPs was 1.64 mg/ml using probit analysis. The concentrations lower than that of IC_{50} of CeO_2 NPs and MPs were used in this study, which was in order to avoid the physical hindrance due to over accumulation of the particles in the culture medium. The cytotoxicity due to loss in cell viability was obvious at 200, 100 and 50 μg/ml of CeO_2 NPs and found to be 76.40%, 77.49% ($p < 0.01$) and 78.68% ($p < 0.05$) respectively as compared to control (100%), while CeO_2 MPs did not significantly decrease the cell viability. In support to our result, **Rosenkranz et al., 2012** also found that CeO_2 NMs were more toxic than their MPs as CeO_2 NMs significantly reduced the mitochondrial metabolism and hence cell viability by MTT assay in H4IIE rat hepatoma cell line when compared to the control. Exposure to CeO_2 NPs for 24 h resulted in a

concentration-dependent increase in LDH leakage and exhibited a significant ($p < 0.01$) cytotoxicity at 100 and 200 µg/ml (Figure 2B). The evident difference between CeO_2 NPs and MPs was noted, as CeO_2 MPs did not induce a significant change. MTT assay showed Co_3O_4 NPs toxicity after 24 hours treatment. Significant inhibition was found ranging from 40-300µg/ml (a=$p<0.05$, b=$p<0.01$), 50-300µg/ml (a=$p<0.05$, b=$p<0.01$) and 50-300µg/ml (a=$p<0.05$, b=$p<0.01$) in HEK293 as shown in Figure 3A. In comparison to its bulk, significant toxic concentration was 300µg/ml ($p<0.05$). LC_{50} calculated using probit analysis software was found to be >300µg/ml. In various studies Co_3O_4 NPs were found cytotoxic and genotoxic. **Papis et al., 2009** revealed that the engineered Co_3O_4 NMs readily entered the cell and caused loss in cell viability when studied with Hep G2 and ECV-302 cell lines. Similarly, **Colognato et al., 2008** showed the genotoxicity of Co NPs in human peripheral leukocytes *in vitro*. The results from LDH membrane leakage assay shows significant increase (Fig. 3B) and hence, toxicity of Co_3O_4 NPs at 100-300µg/ml (a=$p<0.05$, b=$p<0.01$) bulk show no significant toxicity even at 300µg/ml.

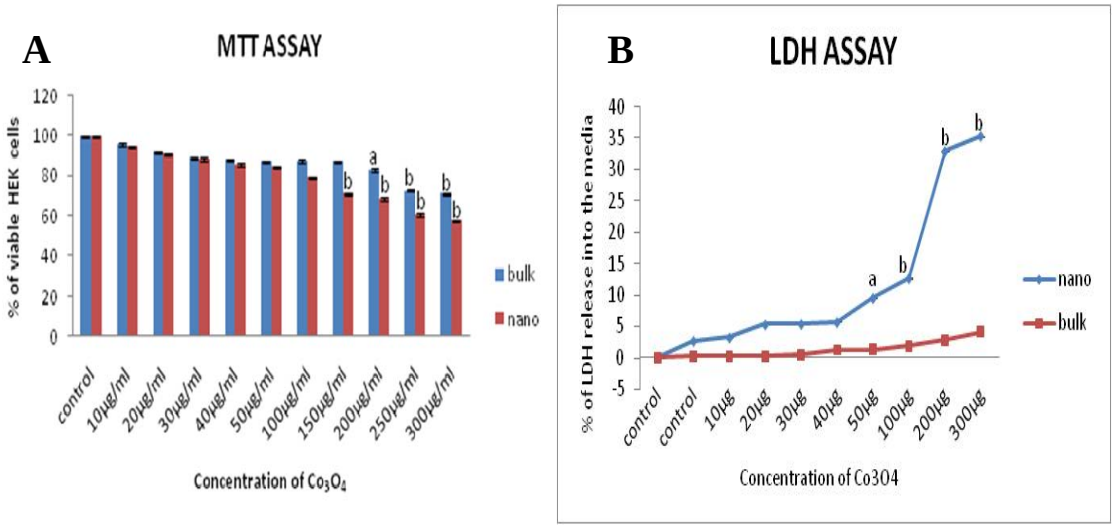
However, WO_3 NPs significantly inhibited viability of cells from 50 - 300 µg/ml in HEK 293 cell lines, whereas WO_3-Bulk significantly inhibited the cell viability from 250 - 300 µg/ml concentrations in HEK 293 cell lines (Fig. 4A).There is negligible work on WO_3 NPs in order to support of our findings. HEK 293 cells were treated with WO_3 NPs and its bulk particles at the concentrations of 0- 300µg/ml for 24h. After incubation cell viabilities in different concentrations were decreased in a dose dependent manner as shown in Figure 4B, similarly its bulk also induced LDH leakage from 30µg/ml onwards.

Fig. 2. MTT and LDH assay of CeO_2 nano and bulk particles in IMR 32 cell line.



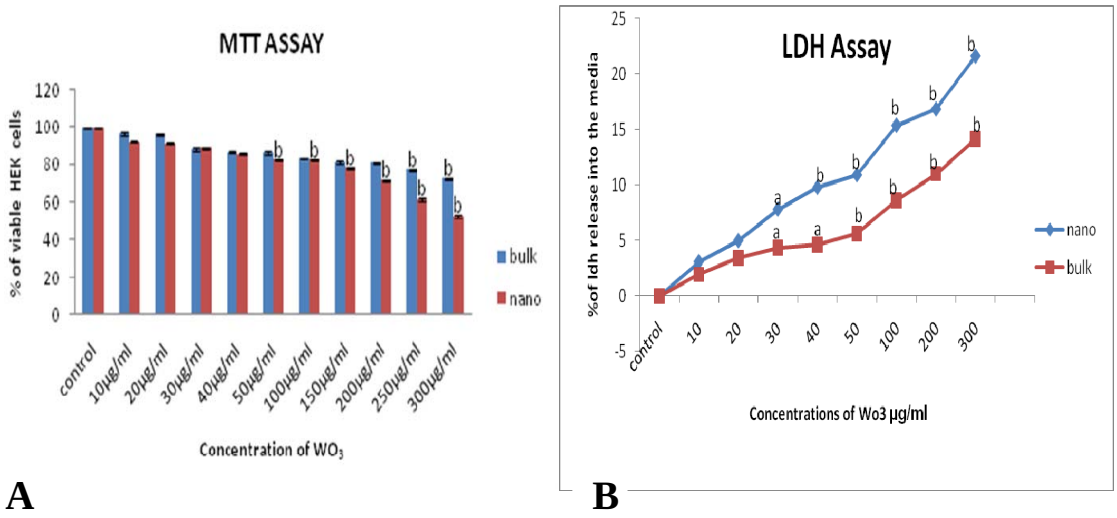
Data represented as mean ± S.D., of 3 replicates per dose, significantly different from control at a=p<0.05, b=p<0.01.

Fig. 3. MTT and LDH assay of Co_3O_4 nano and bulk particles in HEK293 cell lines.



Data represented as mean ± S.D., of 3 replicates per dose, significantly different from control at a=p<0.05, b=p<0.01.

Fig. 4. Comparative effects of WO_3 nano and bulk materials on viability of HEK 293 cell line



Data represented as mean ± S.D., of 3 replicates per dose, significantly different from control at a=p<0.05, b=p<0.01.

3.3. Intracellular release of Reactive oxygen species and hydrogen peroxide

ROS levels were quantified to examine the involvement of oxidative stress in CeO_2 NPs (Fig. 5A). It was noted that CeO_2 NPs induced a significant 1.61 fold and 2.44 fold increase at 100 and 200 µg/ml respectively in ROS levels, whereas CeO_2 MPs did not show a significant change relative to control. Therefore, the production of ROS in IMR32 cells incubated with CeO_2 NPs was much higher than the corresponding MPs exposed cells. CeO_2 NPs significantly increased intracellular H_2O_2 within IMR32 cells at concentrations of 100 ($p < 0.05$) and 200 µg/ml ($p < 0.01$) in comparison to control (Fig. 5B). There was no significant induction in the H_2O_2 levels in cells treated with CeO_2 MPs.

The intracellular ROS generation measured using increase in fluorescence was in dose dependent manner, showed significance from 100 to 300µg/ml (a=p<0.05, b=p<0.01) in cells treated with Co_3O_4 NPs, whereas bulk did not show any significant increase measured by the fluorescence intensity as shown in the Figure 6A.The production of H_2O_2 was found to be significant from 100 to 300µg/ml (a=p<0.05, b=p<0.01) onwards whereas its bulk was significant at 300µg/ml (a=p<0.05, b=p<0.01), as shown in the Figure 6B.Similarly, ROS production was significantly increased from 30µg/ml (p<0.05) of WO_3 NPs where as its bulk 40µg/ml (p<0.05) compared to control as shown in Figure 7A. At the concentration of

200µg/ml fluorescence intensity revealed a 2 fold increase in WO3 NP and its bulk one fold increase at 200µg/ml.

3.4. Nitric oxide production

NO production in IMR32 cells following 24 h exposure to CeO_2 NPs at 0-200 µg/ml is shown in Figure 5C. The NO levels were significantly ($p < 0.01$) increased at 100-200 µg/ml in relation to control. However, in CeO_2 MPs exposed cells there was no significant alteration. The nitrate content used to measure NO using Griess reagent showed significant increase in absorbance in the cells treated with Co_3O_4 NPs from 50 to 300µg/ml ($a=p<0.05$, $b=p<0.01$) onwards whereas its bulk was significant from 200 to 300µg/ml ($a=p<0.05$, $b=p<0.01$), as shown in the Figure 6C. Similarly, WO_3 NPs significantly ($p<0.05$) increased the No levels concentration dependent manner from 50ug/ml and whereas its bulk decreased from 100ug/ml as shown in Figure 7B.

3.5. Lipid peroxidation assay

LPO assay was performed to determine the MDA levels in the IMR32 cell suspension after 24 h treatment with CeO_2 NPs and MPs. A significant increase ($p < 0.01$), in MDA level was observed at 200 µg/ml of CeO_2 NPs, which is evident from Fig. 5D.

3.6. Glutathione content

Alterations in GSH homeostasis can be monitored as an indication of functional damage to cells. IMR32 cells exposed to CeO_2 NPs showed a dose dependent depletion in GSH levels. The exposure concentrations of 100 and 200 µg/ml exhibited statistically significant ($p < 0.01$) depletion of 79% and 76% respectively after 24 h (Fig. 5E).

As there was increase in the ROS generation in the cells treated with Co_3O_4 NPs we measured the cellular GSH a thiol protein, which is essential in maintaining the cellular oxidative status using the Ellman's reagent to support the generation of Oxidative species. We found significant decrease in the levels of GSH in the cells treated with NPs from 50 to 300 µg ($a=p<0.05$, $b=p<0.01$) in cells treated with NMs where as bulk compound did not show any significant decrease in GSH content as shown in Figure 6D.

The intra cellular GSH levels were measured by using DTNB spectroscopic method and were expressed in mg/ml protein. The GSH levels significantly decreased from concentration of

100µg/ml in WO_3 NPs, whereas its bulk from 200µg/ml compared to control as shown in Figure 7C.

3.7. Superoxide dismutase activity

SOD activity in IMR32 cells treated with 100 and 200 µg/ml CeO_2 NPs was significantly ($p < 0.05$, $p < 0.01$ respectively) reduced after 24 h of exposure when compared to unexposed cells as evident from Fig. 5F. However, CeO_2 MPs exposed cell did not exhibit any significant change.The rate of inhibition of pyrogallol auto-oxidation after the addition of enzyme extract was found to be fluctuating around the control cells activity. However, there was no significant increase or decrease in SOD activity even at the highest concentration of the Co_3O_4 NPs. Nevertheless, Co_3O_4 NPs showed decrease in the SOD activity, whereas its bulk showed slightly increase in SOD activity (Fig.6E).

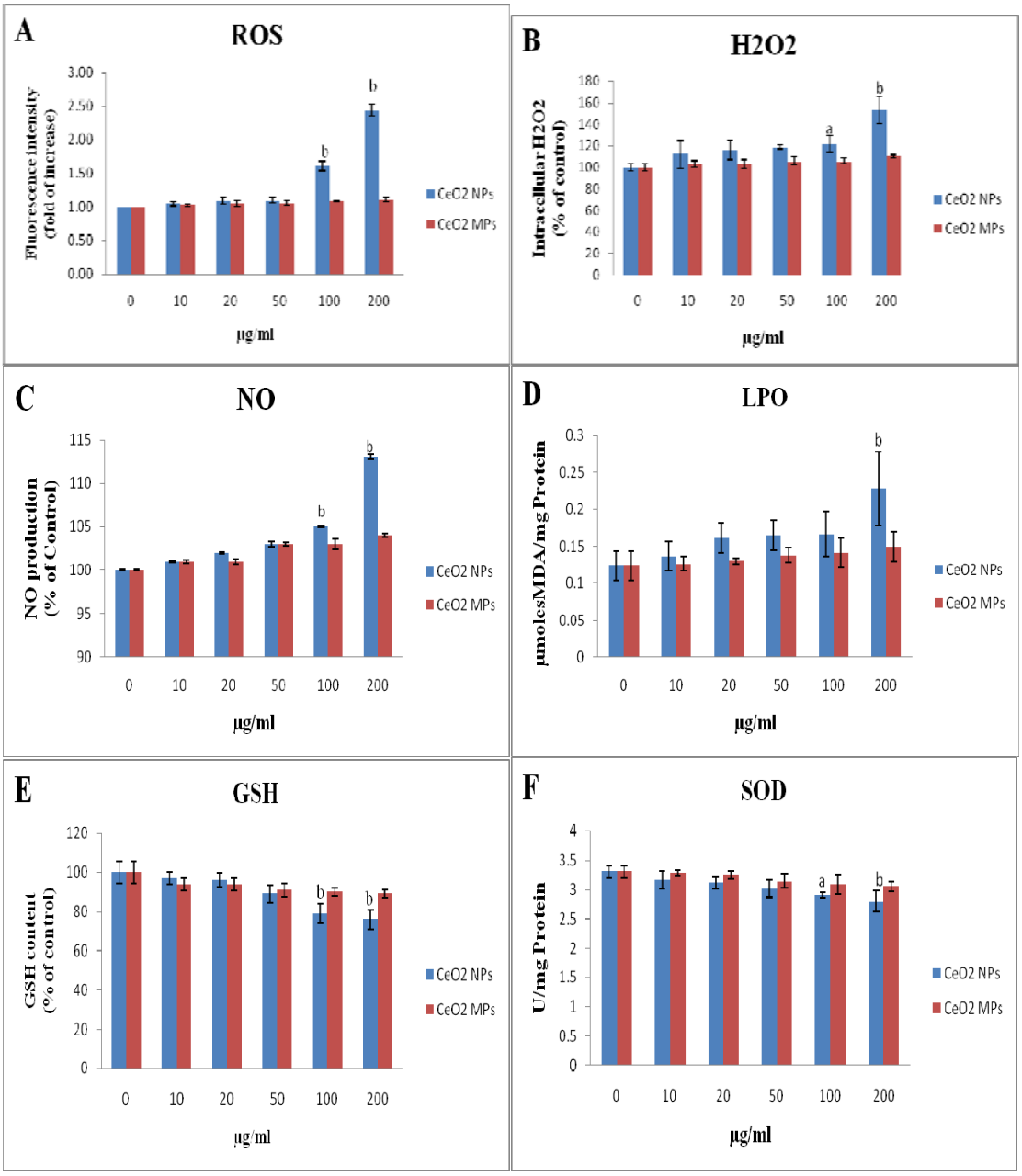
3.8. Catalase activity

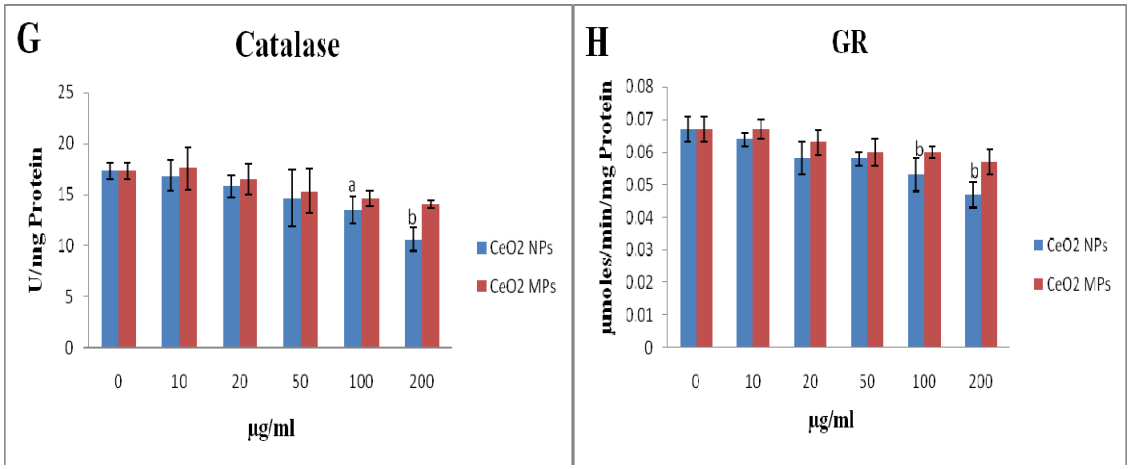
CeO_2 NPs exposed cells at the higher concentrations of 100 and 200 µg/ml revealed significant reduction in CAT levels i.e. 13.48 ± 1.33 and 10.63 ± 1.14 U/mg protein when compared to control (17.31 ± 0.79) at $p < 0.05$ and $p < 0.01$ respectively (Fig. 5G). Cells exposed to CeO_2 MPs did not produce any significant change in CAT levels and were observed to be near to control values. Catalase activity expressed as H_2O_2 decomposed per min per mg protein

3.9. Glutathione Reductase activity

GR activity in IMR32 cells incubated with CeO_2 NPs at concentrations of 100 and 200 µg/ml for 24 h was significantly increased ($p < 0.01$) dose dependently (Fig. 5H). However, CeO_2 MPs did not induce any significant change. GR activity U/mg protein was found to be significant from 100 to 300µg/ml ($a=p<0.05$, $b=p<0.01$) in the cells treated with Co_3O_4 NPs, whereas its bulk was significant at 300µg/ml ($a=p<0.05$, $b=p<0.01$) as shown in the Figure 6F.

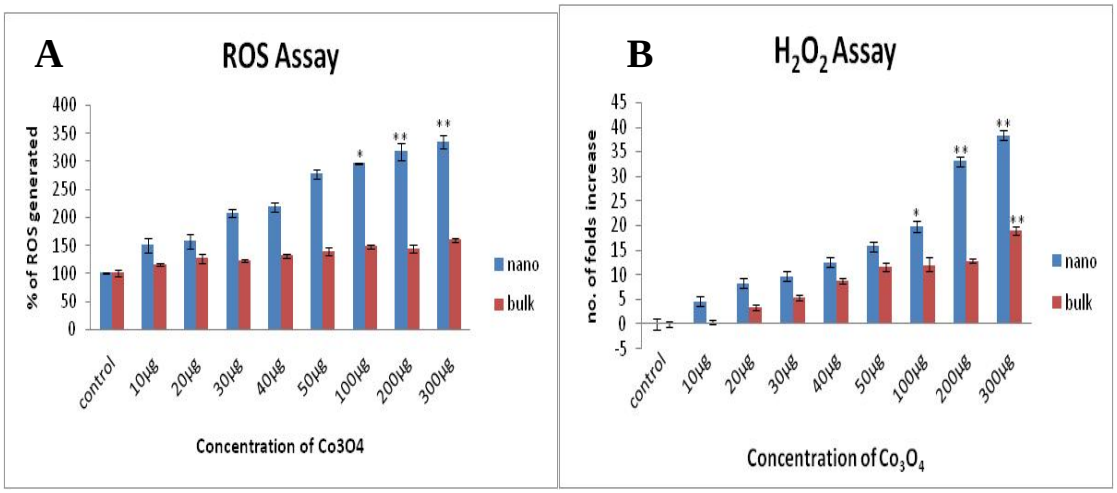
Fig. 5. Effects of CeO_2 NPs and CeO_2 MPs on (A) intracellular reactive oxygen species (ROS), (B) hydrogen peroxide (H_2O_2), (C) nitric oxide (NO), (D) lipid peroxidation (LPO), (E) reduced glutathione (GSH), (F) superoxide dismutase (SOD), (G) catalase, and (H) Glutathione reductase (GR) in IMR32 cells.

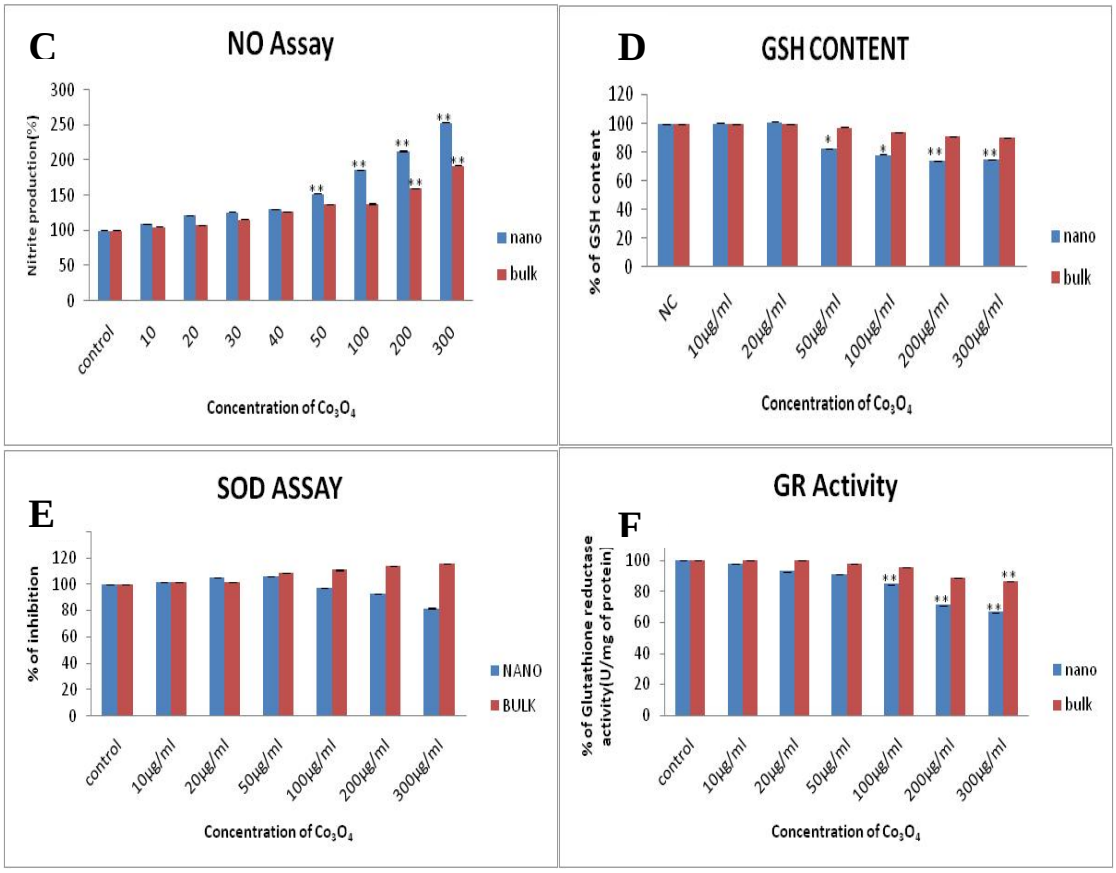




All assays were performed on the culture medium after 24 h incubation with CeO_2 NPs and MPs at concentration of 10, 20, 50, 100 and 200 µg/ml. The control group was treated with media only. Bars indicate the standard deviation from three replicates. The data represented as mean ± SD. Significantly different from control at a = $p < 0.05$, b = $p < 0.01$.

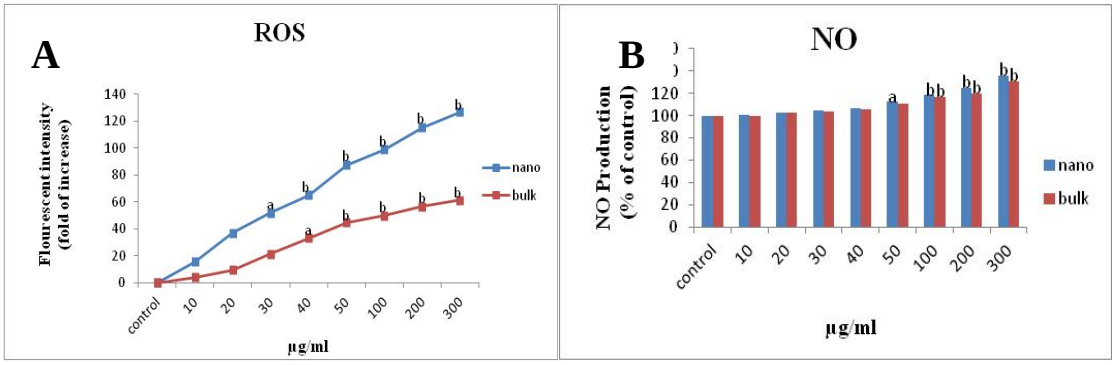
Fig. 6. Effects of Co_3O_4 NPs and Co_3O_4 MPs on (A) intracellular reactive oxygen species (ROS), (B) hydrogen peroxide (H_2O_2), (C) nitric oxide (NO), (D) reduced glutathione (GSH), (E) superoxide dismutase (SOD), (F) Glutathione reductase (GR) in HEK293 cells.

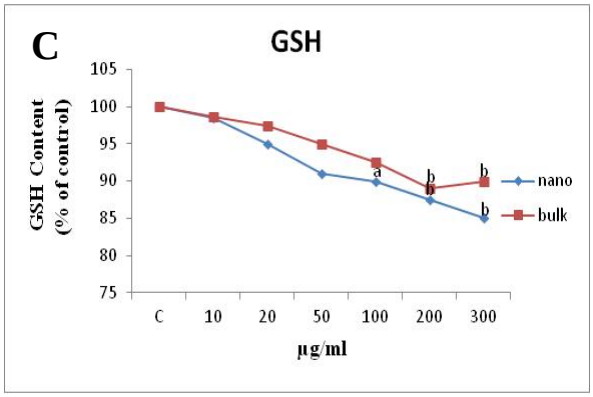




The control group was treated with media only. Bars indicate the standard deviation from three replicates. The data represented as mean ± SD. Significantly different from control at * = $p < 0.05$, ** = $p < 0.01$.

Fig. 7. Effects of WO_3 NPs and WO_3 MPs on (A) intracellular reactive oxygen species (ROS), (B) nitric oxide (NO) and (C) reduced glutathione (GSH) in HEK293 cells.





Data represented as mean ± S.D., of 3 replicates per dose, significantly different from control at a=p<0.05, b=p<0.01.

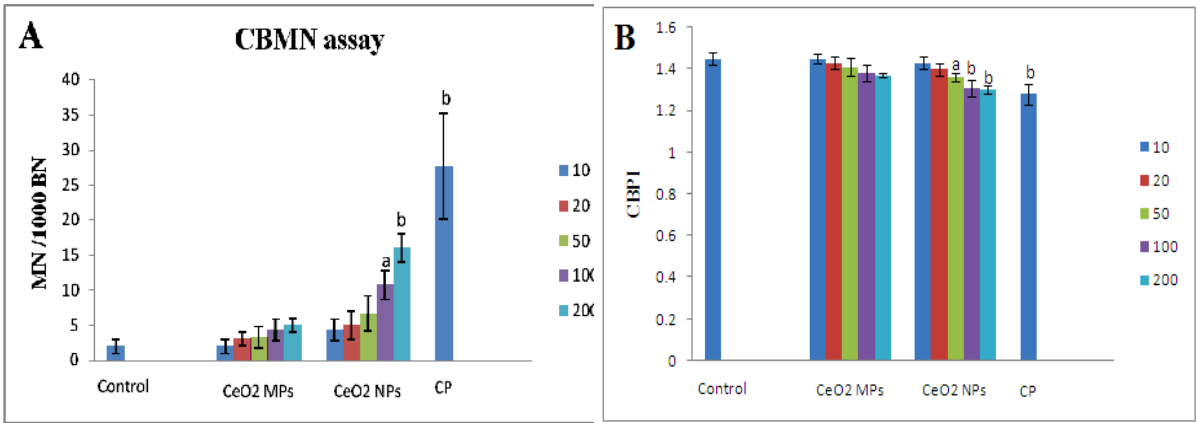
3.10. Cytokinesis-block micronucleus assay

Chromosomal damage induced by the NPs was evaluated using the CBMN assay, in which cell division is blocked to allow the counting of once divided binucleated cells. In the untreated cells, MN frequency was two per 1000 binucleated cells (Fig. 8A). At dose levels of 100 and 200 µg/ml of CeO_2 NPs, the number of MN formed per 1000 binucleated cells were 10.67 ± 2.08 and 16 ± 2 which was significant at $p < 0.05$ and $p < 0.01$ respectively (Fig. 8A). The cell proliferation was assessed during the CBMN assay by the calculation of CBPI. The cell proliferation index was reduced significantly at the doses of 50 µg/ml (1.36 ± 0.02), 100 µg/ml (1.31± 0.04) and 200 µg/ml (1.30 ± 0.02) of CeO_2 NPs in comparison to control (1.45 ± 0.03) (Fig. 8B). Therefore, dose dependent increase in MN frequency and decrease in cell proliferation was recorded upon CeO_2 NPs exposure. Moreover, mono-, bi, and multi- nucleated cells (Fig. 9A) and only a few insignificant number of necrotic cells (Fig. 9B), apoptotic cells (Fig. 9C), and nuclear buds (Fig. 9D) were observed along with MN in binucleated cells (Fig. 9E-H). A significant increase in micronucleus were observed in the cells treated with the Co_3O_4 nano than the cells treated with the bulk at 10 µg/ml (Fig.10) (3 nano,2 bulk), 20µg/ml(5nano ,2bulk), 50µg/ml(9nano,4bulk),100µg/ml (12nano,6bulk) and 200µg/ml(13nano, 7bulk), 300µg/ml (16nano,9bulk) with respect to their controls as shown in the table 2. The CBMN test failed to show significant results with WO_3 NPs and its bulk.

Table 2. CBMN Assay in the HEK293cells treated with the Co_3O_4 NPs and MPs.

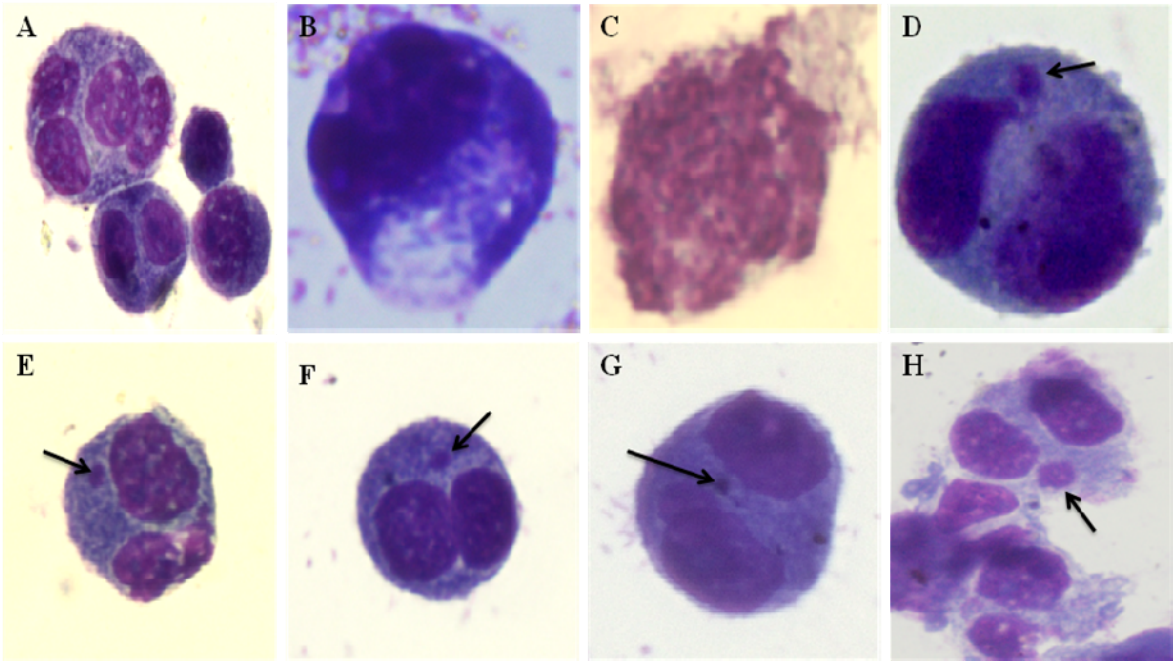
	CBMN INDEX		MICRONUCLEUS	
concentration of Co_3O_4	NANO	BULK	NANO	BULK
control	1.017336	1.017336	2	2
10µg/ml	1.011556	1.293333	3	2
20µg/ml	1.057745	1.412189	5	2
50µg/ml	1.08093	1.637722	9	4
100µg/ml	1.161448	1.795409	12	6
200µg/ml	1.115385	1.792247	13	7
300µg/ml	1.113346	1.892644	16	9

Fig. 8. The genotoxicity of CeO_2 NPs and CeO_2 MPs using in vitro micronucleus test. (A) Frequency of micronucleus in binucleated cells (B) cytokinesis-block proliferation index (CBPI).



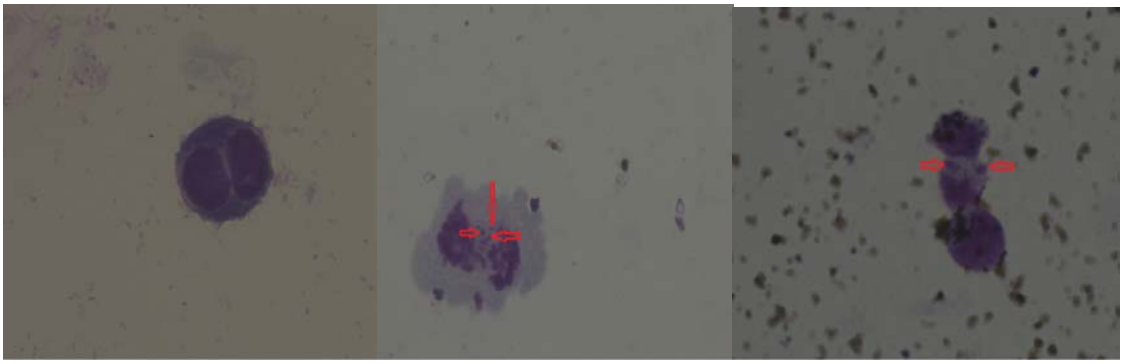
The control group was treated with media only, cyclophosphamide (CP) was used as positive control. Bars indicate the standard deviation from three replicates. The data represented as mean ± SD. Significantly different from control at a = $p < 0.05$, b = $p < 0.01$.

Fig. 9. Photomicrographs of the IMR32 cells scored in the CBMN assay treated with CeO_2 NPs.



(A) Mononucleated cell, binucleated cell and multinucleated cell, (B) necrotic cells , (C) apoptotic cells, (D) binucleated cells containing nuclear buds (indicated by arrow), (E, F, G and H) binucleated cells containing a micronucleus (indicated by arrow).

Fig. 10. (A) HEK 293 Cells without treatment (-ve control), (B) treated with DMSO(+ve control), (C) treated with Co_3O_4 nanoparticles



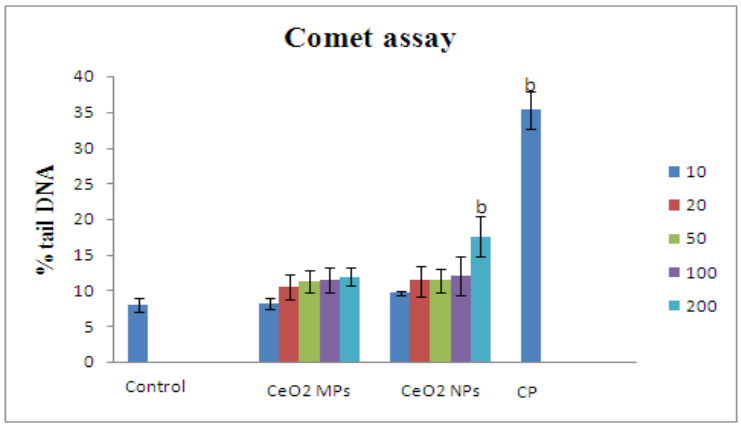
A. B. C.

3.12. **DNA damage**

Comet assay of CeO_2 NPs and MPs treated cells showed a concentration dependent increase in % tail DNA (Fig. 11) when compared to control, which indicates the extent of DNA damage. However, only the highest dose of 200 µg/ml depicted significant ($p < 0.01$) increases in % tail DNA (17.53 ± 2.83). Significant DNA damage was observed in the cells treated with the Co_3O_4 nano from the bulk evident from the study of tail length DNA. The increase in tail length was statistically significant form 100 to 300µg/ml (a=p<0.05, b=p<0.01) in case of cells treated with the NMs and not significant in case cells treated with bulk as shown in the Figure12.

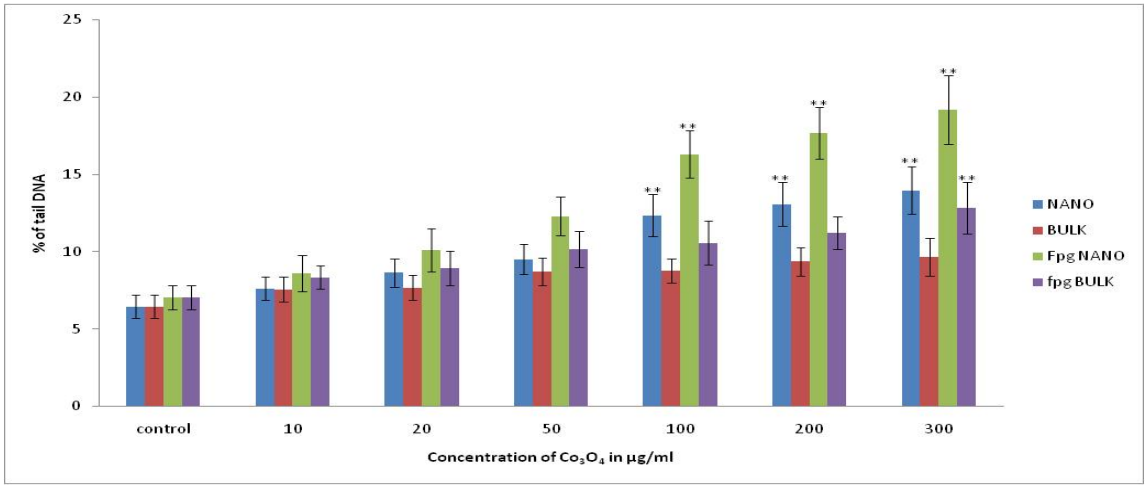
There was significant increase in DNA damage with cells treated with WO_3 NPs, where as there was no significant increase in percentage of tail DNA with WO_3 bulk particles.

Fig. 11. Mean % tail DNA in IMR32 cells after 24 h exposure of different doses of CeO_2 NPs and MPs.



Bars indicate the standard deviation from three replicates. The data represented as mean ± SD. Significantly different from control at a = $p < 0.05$, b = $p < 0.01$.

Fig. 12. Comparison of tail length of HEK-293 cell lines treated with Co_3O_4 nano and bulk material.



Bars indicate the standard deviation from three replicates. The data represented as mean ± SD. Significantly different from control at * = $p < 0.05$, ** = $p < 0.01$.

Additional work:

In addition to the above reported *in vitro* work we have completed the study with these NPs in *in vivo* system (Albino Wistar rats). The in vivo work with CeO_2 NPs has been completed and data has been published. However, studies with Co_3O_4 and WO_3 NPs are going on along with paper publications.

Further studies to be done in the future if funds are available:

- Cellular uptake and quantitative determination in cells
- Apoptotic assay in cells.
- Real time reverse transcriptase polymerase chain reaction (RT-PCR) using stress and toxicity array analysis.

Conclusion:

Toxicological effects were observed due to the treatment of these NMs and their Bulk materials. These alterations were mostly dose and size dependent i.e. higher dose showed more effect compare to lower dose. Moreover, bulk materials were least toxic in causing

toxicity, suggesting that particle size of the NMs play vital role in causing the toxicity. Our findings suggest that these NMs could be relatively safe. However, more studies are warranted for careful assessment to ensure safety of these NMs in various applications.

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Publications:

1. **Monika Kumari**, Shailendra Pratap Singh, Srinivas Chinde, Mohammed Fazlur Rahman, Mohammed Mahboob and Paramjit Grover. Toxicity study of cerium oxide nanoparticles in human neuroblastoma cells. International Journal of Toxicology 2014 Mar;33(2):86-97 **I.F. 1.346**

2. **Monika Kumari**, Srinivas Indu Kumari, and Paramjit Grover. Genotoxicity analysis of cerium oxide micro and nanoparticles in Wistar rats after 28 days of repeated oral administration. Mutagenesis 2014 Nov;29(6):467-79.**I.F. 3.497**

3. **Monika Kumari**, Srinivas Indu Kumari, Sarika Srinivas Kalyan Kamal, Paramjit Grover. Genotoxicity assessment of cerium oxide nanoparticles in female Wistar rats after acute oral exposure. Mutation Research (Early online). **I.F. 2.22**

Thesis submitted

- Thesis entitled "**Genotoxicity Assessment of Cerium Oxide Nanoparticles: An *in vitro* and *in vivo* Study**" has been submitted out of this project to the Genetics Department, Osmania University, Hyderabad, India by **Monika Kumari** under the guidance of Dr Ms. Paramjit Grover