

Mechanistic investigation of toxicity of chromium oxide nanoparticles in murine fibrosarcoma cells

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Abstract: Chromium oxide nanoparticles ($\text{Cr}_2\text{O}_3\text{NPs}$) are widely used in polymers and paints. In the present study, we aimed to determine the toxicity of $\text{Cr}_2\text{O}_3\text{NPs}$ in murine fibrosarcoma (L929) cells. The cytotoxicity of $\text{Cr}_2\text{O}_3\text{NPs}$ was measured by MTT and neutral red uptake assays; $\text{Cr}_2\text{O}_3\text{NPs}$ had significant cytotoxic effects on L929 cells. Enhancement of intracellular reactive oxygen species was observed in L929 cells after exposure to $\text{Cr}_2\text{O}_3\text{NPs}$. $\text{Cr}_2\text{O}_3\text{NPs}$ produced caspase-3, indicating that exposure to $\text{Cr}_2\text{O}_3\text{NPs}$ induced apoptosis. After exposure to $\text{Cr}_2\text{O}_3\text{NPs}$, the cellular glutathione level decreased and lipid peroxidation, superoxide dismutase, and catalase increased in a dose- and time-dependent manner. By using single-cell gel tests, we also observed increased DNA damage in a $\text{Cr}_2\text{O}_3\text{NP}$ exposure-duration- and dose-dependent fashion. Cell toxicity and DNA damage may be useful biomarkers for determining the safety of $\text{Cr}_2\text{O}_3\text{NPs}$ in human and animal health.

Keywords: $\text{Cr}_2\text{O}_3\text{NPs}$, L929 cells, MTT assay, oxidative stress

Introduction

Engineered nanoparticles are extensively used in the industrial sector. Chromium oxide nanoparticles ($\text{Cr}_2\text{O}_3\text{NPs}$) are used in pigments, thermal protection coating, and sensing of humidity.^{1,2} It has been reported that interaction of nanoparticles with biological molecules depends on their shape and size.^{3,4} To use nanoparticles for therapeutic applications, it is important to assess their toxicity in cancer cells and normal cells. Nanoparticles can induce effects that are harmful to human health, specifically depending on the particle size.⁵ Warheit et al⁶ and Oberdorster et al⁷ reported that oxidative stress and inflammation occur in animals upon inhalation of nanoparticles. The toxic effects of $\text{Cr}_2\text{O}_3\text{NPs}$ (eg, DNA damage) are of specific concern because alterations in genetic materials have the potential to cause cell death, cancer, and adverse reproductive effects. The comet assay is a widely accepted technique used to determine the genotoxicity of xenobiotic compounds in living organisms and to determine the toxic potential of different doses of carcinogens or genotoxic materials in animals.⁸ In vitro examinations have validated the physiological response to nanoparticles in animals and furnished results demonstrating increased oxidative stress in nanoparticle-treated cell lines.^{9,10}

The present study was conducted to assess the toxicity of $\text{Cr}_2\text{O}_3\text{NPs}$ in murine fibrosarcoma (L929) cells. We measured levels of reactive oxygen species (ROS), superoxide dismutase (SOD), glutathione (GSH), and malondialdehyde (MDA) in L929 cells in response to $\text{Cr}_2\text{O}_3\text{NPs}$. DNA damage effects were evaluated using the comet assay.

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Materials and methods

Chemicals and reagents

Cr₂O₃NPs; 2',7'-dichlorofluorescein diacetate (DCFH-DA); 5,5'-dithiobis(2-nitrobenzoic acid) and GSH were acquired from Sigma-Aldrich (St Louis, MO, USA).

Characterization of Cr₂O₃NPs

Cr₂O₃NP was suspended in culture media (1 mg/mL) and dispersed by using a sonicator for 20 minutes with 40 W power, at normal temperature. Size and shape characterization of Cr₂O₃NPs was studied using high-resolution transmission electron microscope (HRTEM). Size of Cr₂O₃NP in DMEM was examined by using dynamic light scattering (DLS) (Nano-Zeta Sizer-HT, Malvern Instrument, Malvern, UK).

L929 cell culture and treatment

Murine fibrosarcoma (L929) cell line was obtained from American Type Culture Collection (Rockville, MD, USA). L929 cells with 80% growth were collected and cultured into 96-well plates and petri dishes. We treated L929 cells to various doses (0, 10, 50, and 100 µg/mL) for 12 and 24 hours.

Morphological analysis of cells

Morphology of L929 cell lines were seen under an inverted microscope (Leica DMIL, Leica Microsystem, Wetzlar, Germany) after Cr₂O₃NPs treatment for 24 hours.

MTT assay

Viability of L929 cells was assessed after treatment of Cr₂O₃NP by MTT assay.¹¹ Cell viability was calculated using the following formula:

$$\text{Cell viability (\%)} = \frac{\text{OD exposed cells} - \text{OD of blank}}{\text{OD of control} - \text{OD of blank}} \times 100$$

NRU assay

Viability of L929 cells after treatment of Cr₂O₃NP for 12 and 24 hours was assessed by neutral red uptake (NRU) assay.¹²

Measurement of ROS generation

Generation of ROS was measured in L929 cells after exposure of Cr₂O₃NPs for 12 and 24 hours using DCFH-DA dye.¹³

Measurement of oxidative biomarkers

L929 cells (~6×10⁶) were grown in a flask (75 cm²) and administered Cr₂O₃NPs (0, 10, 50, and 100 µg/mL) for 12 and 24 hours. Exposed L929 cells were collected and washed with chilled phosphate-buffered saline. The collected cells were lysed with a lysing solution. The cells and the lysing

solution were cryocentrifuged at 10⁴× g for 10 minutes at 4°C, following which they were placed on ice for oxidative stress. Protein content in cell lysate was determined by using Lowry et al's method.¹⁴

Lipid peroxide (LPO) was evaluated by estimating the production of MDA.¹⁵ SOD level was measured according to Alarifi et al's method.¹⁶ GSH level was evaluated by using Elman's methods.¹⁷

Condensation of chromosome and caspase-3 activity

We observed condensed chromosome in L929 cells by using Hoechst 33342 (Thermo Fisher Scientific) staining after exposure to Cr₂O₃NPs for 12 and 24 hours. Images of condensed chromosome were captured by fluorescence microscopy (Nikon, Tokyo, Japan). Caspase-3 enzyme was quantified by using Alarifi and Ali's method.¹⁸

Comet assay

DNA damage was assessed by comet technique according to Ali et al¹² method.

Data analysis

The data was presented as average ± standard error (SE). Data were analyzed by one-way analysis of variance (ANOVA), followed by the Dunnett's test. Statistical significance was denoted as *P*<0.05.

Results

Size of Cr₂O₃NPs

The size of the Cr₂O₃NPs used in this work was determined using HRTEM. Most of the nanoparticles were spherical, but some of them were polygonal. The mean diameter of the Cr₂O₃NPs was 36.8 nm. Table 1 shows the hydrodynamic diameter and zeta potential of the Cr₂O₃NPs in DMEM.

Morphological alteration of L929 cells

Figure 1 shows the shape and size of untreated L929 cells (Figure 1A) and cells exposed to Cr₂O₃NPs for 24 hours (Figure 1B). L929 cells became spherical and separated from the surface of the culture plates after exposure to 100 µg/mL of Cr₂O₃NPs (Figure 1B).

Table 1 DLS measurements of Cr₂O₃NPs in culture media

DLS measurements	Value
Hydrodynamic size	253±4.0 nm
Zeta potential	-13.6 mV

Abbreviations: Cr₂O₃NPs, chromium oxide nanoparticles; DLS, dynamic light scattering.

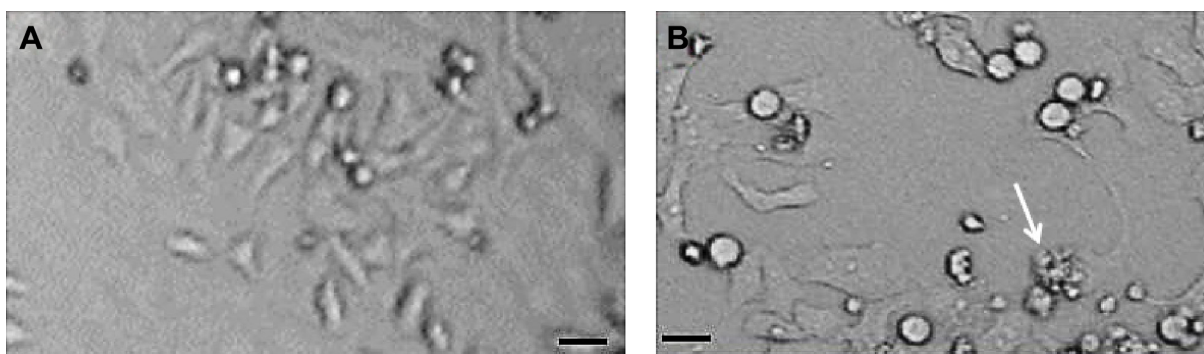


Figure 1 Morphology of L929 cells.

Notes: (A) Control. (B) At 50 µg/mL of Cr₂O₃NPs for 24 hours. Scale bar (—) is 50 µm; magnification 40×. The arrow indicates a damaged cell.

Abbreviation: Cr₂O₃NPs, chromium oxide nanoparticles.

Cytotoxicity

Cr₂O₃NP-induced cytotoxicity in L929 cells was measured by the MTT and NRU assays. Both MTT (Figure 2A) and NRU (Figure 2B) results indicated a dose- and time-dependent cytotoxicity after exposure of L929 cells to Cr₂O₃NPs.

Oxidative stress and production of ROS

Induction of oxidative stress by Cr₂O₃NPs was evaluated. Cr₂O₃NPs generated ROS in duration and concentration basis (Figure 3A–C). Due to exposure of Cr₂O₃NP, GSH was decreased (Figure 4B) and LPO, SOD and catalase were increased in L929 cells (Figure 4A, C, and D).

Caspase-3 activity and condensation of chromosome

Chromosome lesions were observed in L929 cells after treatment with Cr₂O₃NPs (Figure 5A and B). On treatment with Cr₂O₃NPs, the level of caspase-3 raised (Figure 5C).

DNA damage

Damage of DNA was determined in tail DNA (%) (Figure 6A) and Olive tail moment (Figure 6B) in untreated and Cr₂O₃NP-treated L929 cells. L929 cells exhibited more DNA damage after exposure to Cr₂O₃NPs (Figure 6D) than control group (Figure 6C). Maximum level of DNA damage was recorded in L929 cells exposed to 100 µg/mL of Cr₂O₃NPs for 24 hours (Figure 6).

Discussion

Metal nanoparticles are widely used in industry and medicine, but their applications have been limited because of significant toxicity and chronic secondary effects. It is the physiochemical properties of nanoparticles such as size, surface area, and structure that make them useful in nanomedicine.¹⁹ Shi and Dalal²⁰ reported that chromium shows toxicity due to conversion of chromium(IV) to chromium(III) as a consequence of OH· generation. Chromium(IV) crosses plasma membranes through ion channels.

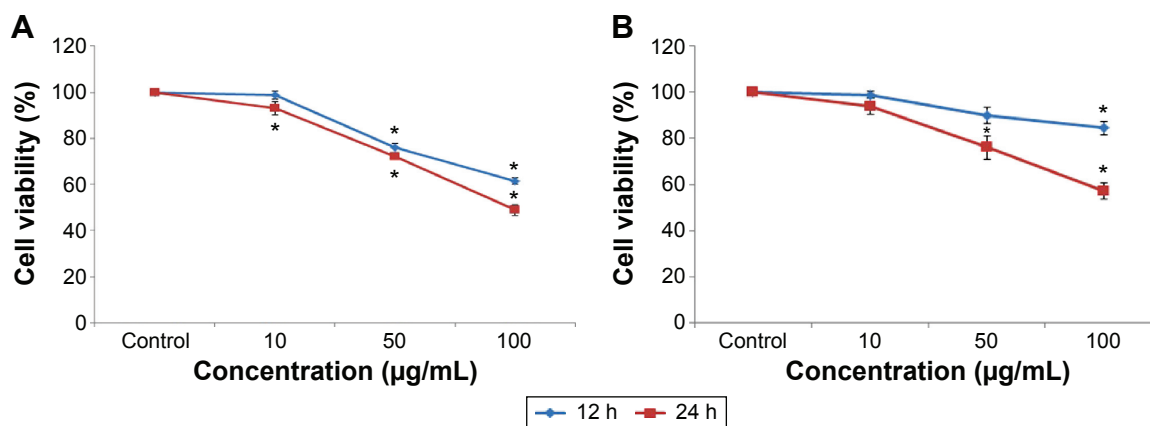


Figure 2 Cytotoxicity of Cr₂O₃NPs in L929 cells for 12 and 24 hours.

Notes: (A) MTT and (B) NRU tests. **P* < 0.05 vs control.

Abbreviations: Cr₂O₃NPs, chromium oxide nanoparticles; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; NRU, neutral red uptake; h, hours.

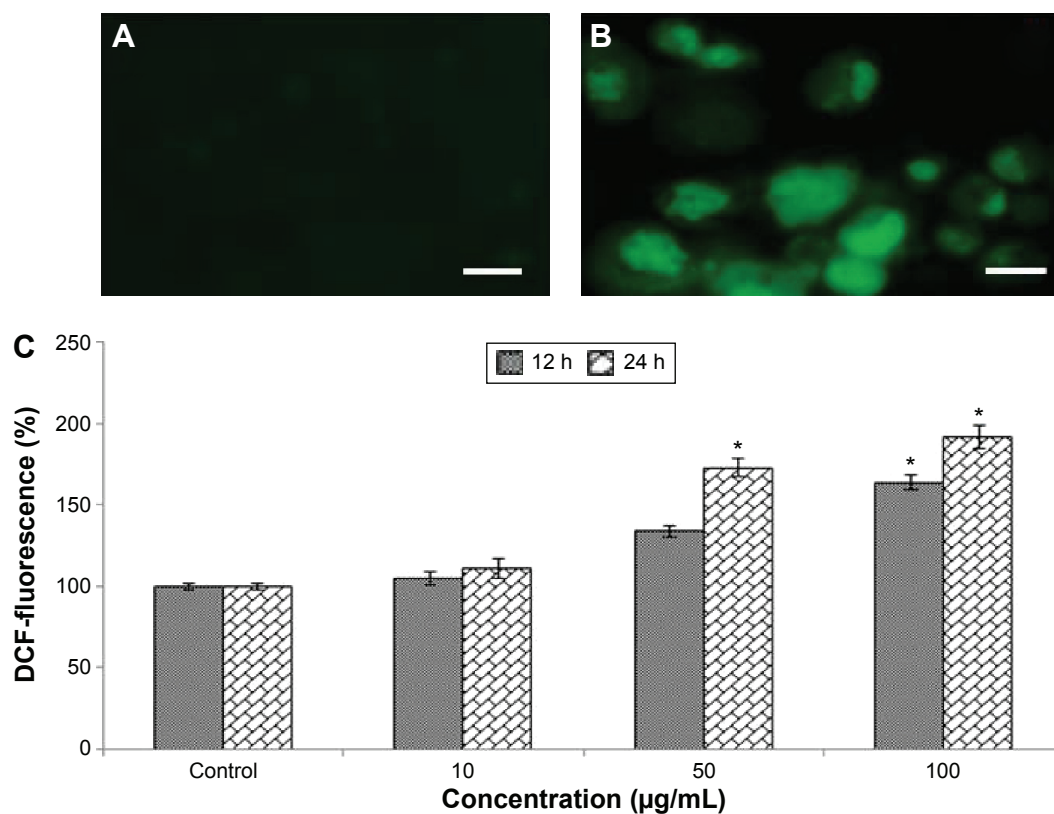


Figure 3 Cr₂O₃NPs induced ROS in L929 cells.

Notes: (A) Control (B) at 100 µg/mL of Cr₂O₃NPs for 24 hours (C) % ROS generation due to Cr₂O₃NPs in L929 cells. Scale bar (—) 50 µm; magnification 40×. *P<0.05 vs control.

Abbreviations: Cr₂O₃NPs, chromium oxide nanoparticles; DCF, dichlorofluorescein; ROS, reactive oxygen species.

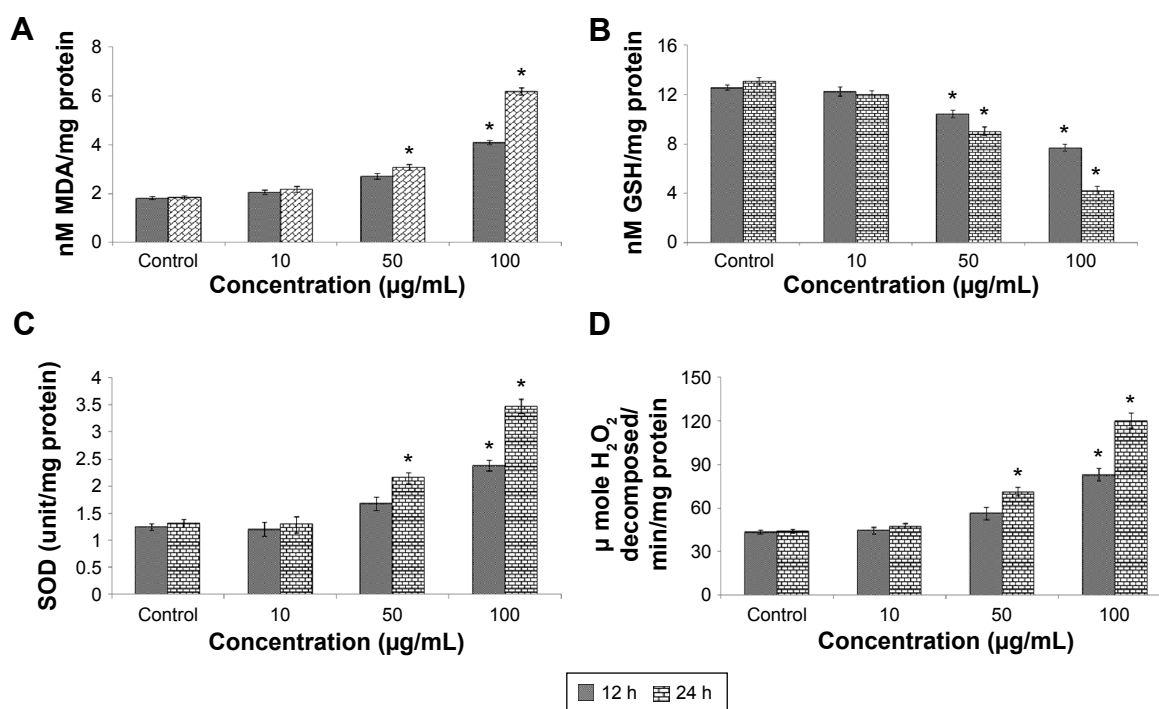


Figure 4 (A) LPO, (B) GSH, (C) SOD, and (D) catalase in L929 cells after exposure to Cr₂O₃NPs for 12 and 24 hours.

Note: *P<0.05 vs control.

Abbreviations: Cr₂O₃NPs, chromium oxide nanoparticles; GSH, glutathione; LPO, lipid peroxidation; SOD, superoxide dismutase; h, hours.

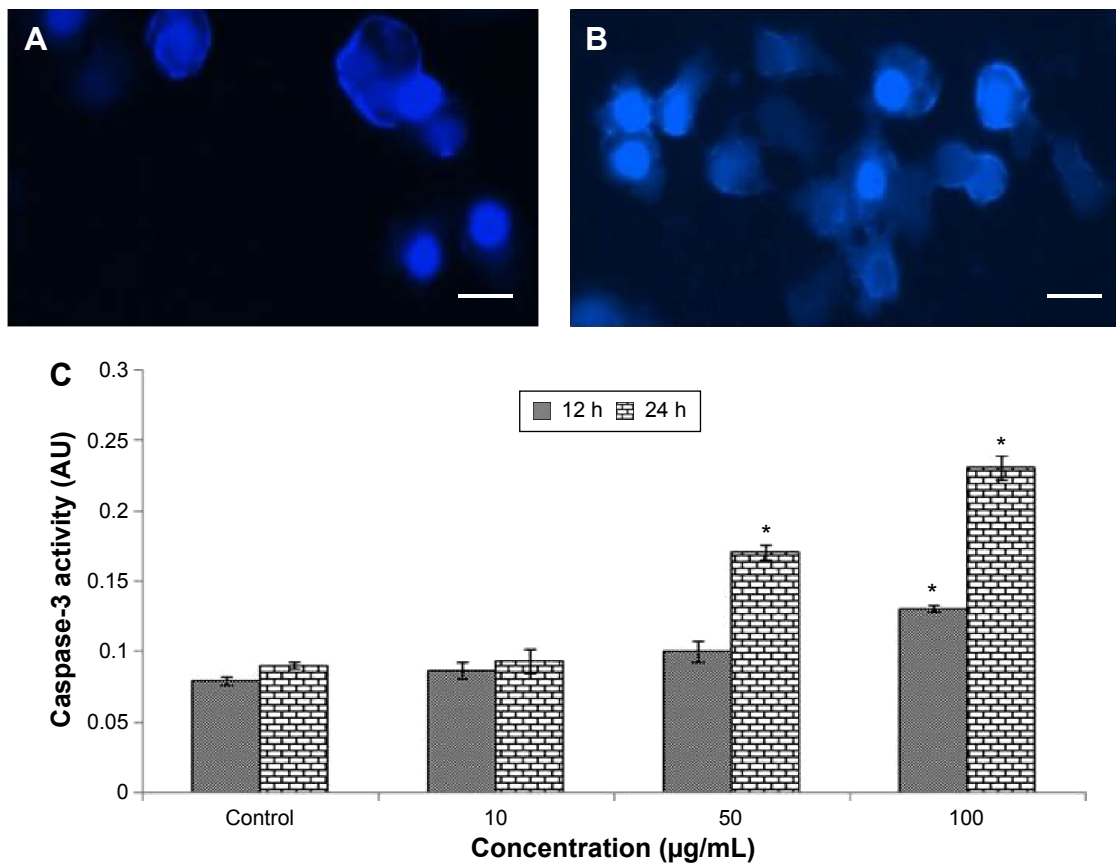


Figure 5 Chromosomal condensation and caspase-3 activity in L929 cells after treatment with Cr_2O_3 NPs.

Notes: (A) Control, (B) at 50 µg/mL of Cr_2O_3 NPs for 24 hours. (C) Caspase-3 activity. * $P < 0.05$ vs control. Scale bar (—) 50 µm; magnification 40×.

Abbreviations: AU, arbitrary units; Cr_2O_3 NPs, chromium oxide nanoparticles; h, hours.

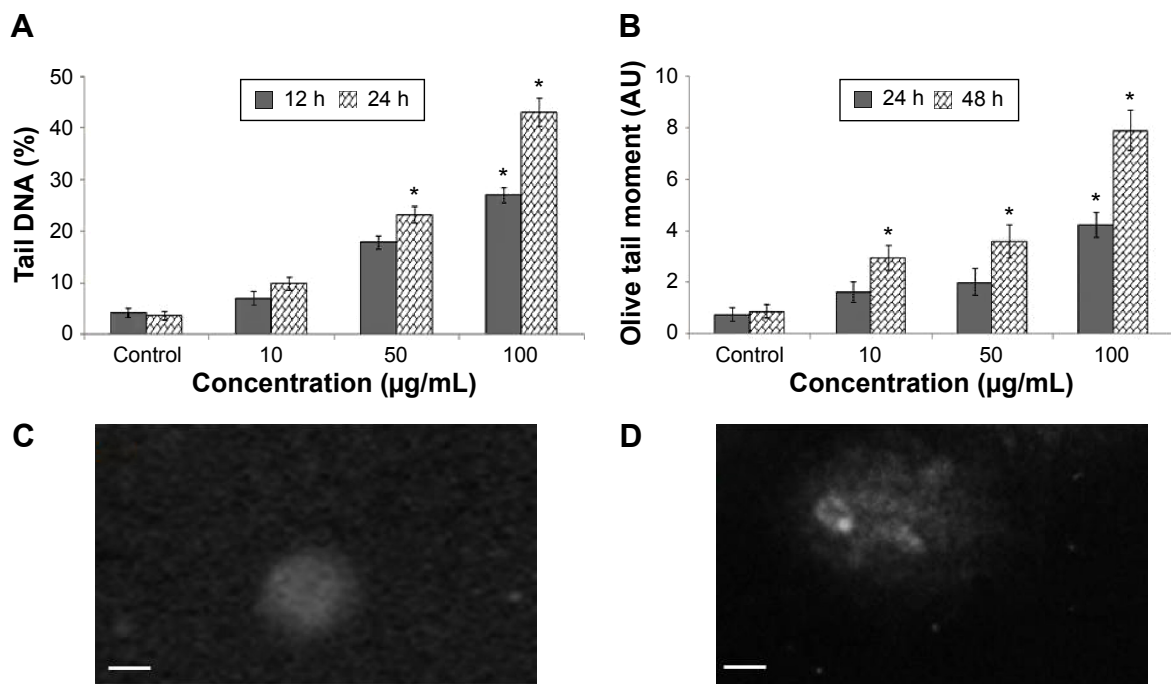


Figure 6 DNA lesions in L929 cells because of Cr_2O_3 NPs.

Notes: (A) Tail DNA (%). (B) Olive tail moment. (C) Control cell. (D) At 100 µg/mL of Cr_2O_3 NPs for 24 hours. Scale bar (—) is 50 µm; magnification 40×. * $P < 0.05$ vs control.

Abbreviations: Cr_2O_3 NPs, chromium oxide nanoparticles; h, hours.

Here, we measured the size and zeta potential of Cr₂O₃NPs by TEM and DLS, respectively, although the size determined by DLS was larger than that quantified by TEM. Our results demonstrate that toxicity of Cr₂O₃NPs in L929 cells was time- and dose-dependent, as measured by the cellular reduction of MTT and uptake of neutral red dye. The MTT test is a biomarker for activity of succinate dehydrogenase, a main constituent of the mitochondrial electron transport chain, and is used to measure cell survival; only live cells with undamaged mitochondria can reduce MTT dye, and the total quantity of MTT reduced is decreased in a ratio of the number of live cells. After 12 hours of exposure of cells to Cr₂O₃NPs, there was relatively little effect seen in the MTT test. However, after 24 hours of treatment, significant dose-dependent reduction in MTT levels were observed. The toxic effects of Cr₂O₃NPs were validated by the observation of separation of dead cells from the surface of culture plates.

Xia et al²¹ reported that the production of ROS is a possible mechanism of Cr₂O₃NP toxicity, which might induce DNA damage. Cr₂O₃NPs were found to induce production of intracellular ROS when assessed by H₂DCFDA, thus it could be initial action for its toxic effect in L929 cells. Cr₂O₃NPs contain a transition metal capable of initiating Fenton reactions. The ROS-producing capability of Cr₂O₃NPs may lead to various impairments of DNA such as mutations and oxidative damage. Elevation of LPO was observed in L929 cells due to Cr₂O₃NPs. GSH levels in cells declined, while catalase and SOD increased in a time- and dose-dependent manner on exposure to Cr₂O₃NPs. Single-cell gel tests demonstrated significant enhancement in percentage tail DNA and Olive tail moment, both markers of DNA fragmentation, at higher doses of Cr₂O₃NPs after exposure for 24 hours.

We observed a correlation between ROS generation and fall in GSH, suggesting that greater generation of ROS induced LPO with a simultaneous decline in glutathione to hunt those free radicals. Kang et al²² reported that oxidative stress and LPO lead to apoptosis and DNA damage. During apoptosis, a chain of biochemical reactions occurs, inducing alteration of L929 cell shape and cell death.

Kroemer et al²³ reported some biomarkers to characterize apoptosis, including DNA fragmentation, cell membrane blebbing, condensation of chromosome, and shrinking and disintegration of organelles. In the present study, Hoechst 33342 (Thermo Fisher Scientific) staining of Cr₂O₃NPs-treated L929 cells indicated nuclear fragmentation and condensation. Chen et al²⁴ reported that, because of their small size and shape, nanoparticles interact with DNA. DNA damage can either induce cell death or carcinogenesis,

consequently disrupting normal cell functions. We found DNA-damaging effects of Cr₂O₃NPs in L929 cells by using the comet test, which identifies double- and single-strand breaks at minute damage of DNA.²⁵

This study indicates that use of manufactured Cr₂O₃NPs should be cautiously evaluated because of its hazardous effects on animals.

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Disclosure

The authors report no conflicts of interest in this work.

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