Size-Dependent Toxicity of Dioxide Manganese Particles on DNA Damage in Hela Cells

MAO Cai-xia¹, YANG Guang-tao¹, QIAO Yong-kang¹, LI Yan¹, XI Zhu-ge²*, YANG Xu¹,#

1. Laboratory of Environmental Science and Hubei Key Laboratory of Genetic Regulation and Integrative Biology, College of Life Science, Huazhong Normal University, Wuhan 430079, China
2. Institute of Health and Environmental Medicine, Academy of Military Medical Sciences, Tianjin 300050, China

Abstract: A comparison was made for the size-dependent DNA damage induced by both nano dioxide manganese particles (Nano-MnO₂) and normal size dioxide manganese particles (Nor-MnO₂). Hela cells were exposed to different concentrations of both Nano-MnO₂ and Nor-MnO₂ (0, 100, 200, 400μg·mL⁻¹), respectively. The DNA damage of Hela cells was measured by the comet assay after 24h MnO₂-cultivated. Results showed that the Tail DNA% and Tail Moment were both significantly increased after exposed to both Nano-MnO₂ and Nor-MnO₂ when compared with the control (p<0.01 for all). At the same concentration level, Nano-MnO₂ could induce more serious DNA damage than Nor-MnO₂ (p<0.01 for all). The results suggested that Nano-MnO₂ should have more serious toxic effect regarding to DNA damage of the Hela cells than that of Nor-MnO₂.

Keywords: nano dioxide manganese particles (Nano-MnO₂); normal size dioxide manganese particles (Nor-MnO₂); DNA damage; single-cell gel electrophoresis (SCGE, comet assay); Hela cells

二氧锰颗粒对 Hela 细胞 DNA 损伤的尺度依赖性毒作用

毛彩霞¹，杨光涛¹，乔永康¹，李岩¹，袁著革²*，杨旭¹，#

1. 华中师范大学生命科学学院 环境科学实验室和遗传调控与整合生物学湖北省重点实验室，武汉 430079
2. 军事医学科学院卫生和环境医学研究所 环境毒理学研究室，天津 300050

摘要：为了观察二氧化锰颗粒物所导致的尺度依赖性 DNA 损伤作用，将纳米尺度二氧化锰颗粒物 (Nano-MnO₂) 和常规尺度二氧化锰颗粒物 (Nor-MnO₂) 所致的 DNA 损伤进行了对比研究。将 Hela 细胞分别暴露于不同浓度 (0, 100, 200, 400μg·mL⁻¹) 的 Nano-MnO₂ 和 Nor-MnO₂ 中, 染毒 24h, 采用彗星实验检测 Hela 细胞的 DNA 损伤水平。结果表明；与对照组相比，Nano-MnO₂ 和 Nor-MnO₂ 均可使彗尾 DNA 百分比 (Tail DNA%) 和尾矩 (Tail Moment) 显著增加 (p<0.01)；而在同一浓度水平上，Nano-MnO₂ 所致的 DNA 损伤则比 Nor-MnO₂ 所致的 DNA 损伤更为严重 (p<0.01)。结果提示：二氧化锰颗粒物对 Hela 细胞 DNA 损伤具有尺度依赖性毒作用，纳米尺度比常规尺度二氧化锰颗粒物毒作用更强烈。

关键词：纳米尺度二氧化锰颗粒物；常规尺度二氧化锰颗粒物；DNA 损伤；彗星实验；Hela 细胞

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Biography MAO Cai-xia (1982—), female, postgraduate; *Corresponding author, E-mail: xzg@tj.edu.cn; # Corresponding author, E-mail: yangxu@mail.ccnu.edu.cn
1 Introduction

The present occupational exposure limit is based on the mass concentration of particles but not size, which takes no account of the likely enhanced toxicity of ultrafine particles in exposed workers. Many properties of the macro-material are altered essentially when it degrades into nano-particles and defined as particles in the size range <100nm. At the same time, the biological effect may change basically. For instance, particulate titanium dioxide (TiO_2) is classified as a nuisance dust and so is considered to have few adverse effects on the lung except at high exposure concentration. Nevertheless, there are now reports showing that ultrafine titanium dioxide (Nano-TiO_2) with a diameter of 20nm can induce a more serious lung inflammatory reaction than 250nm TiO_2 particles (Ferin et al., 1992; Zhang et al., 2000). This report has changed the popular concept about the particle toxicity: the fine particles, even though non or low-toxic, will exhibit high toxic effect at a ultrafine diameter. A study investigating the association between fine and ultrafine particulate air pollution and cardio respiratory health indicates that some cardiovascular and respiratory symptoms are related to levels of particulate air pollution among medicated subjects with coronary heart disease. The results indicate that the associations are stronger for fine than for ultrafine particles and gaseous air pollutants (Hartog et al., 2003). Moreover, pulmonary toxicity studies in rats demonstrate that nanoparticles produce enhanced inflammatory responses when compared to larger sized particles of identical chemical composition at equivalent mass concentrations (Oberdörster et al., 2000).

MnO_2, a multi-purpose fine inorganic material with a wide range of uses, can be used as molecular sieves and senior catalyst. Additionally, Nano-MnO_2 is the leader candidate of the rechargeable batteries for its superior ion-electron conductivity and higher potential (Yang et al., 2006). At the same time, increasing attention has been gained on its adverse effect on human beings for its widely application. It is expected that Nano-MnO_2 may be more toxic than Nor-MnO_2 on account of its smaller size and larger surface; however, it has not been well investigated yet. In this study, we used two sizes of MnO_2 particles for the exposures and detected the DNA damage effect in Hela cells with single-cell gel electrophoresis (SCGE, comet assay), to evaluate the genotoxicity of Nano-MnO_2 and to investigate the possible mechanism.

2 Materials and methods

2.1 Reagents and apparatus

RPMI 1640 and fetal bovine serum (FBS) were purchased from Gibco. Triton X-100 was purchased from Amresco. Normal melting agarose and low melting agarose were purchased from Promega. Cell counting 8 kits was purchased from Dojindo.

Apparatus include CO_2 incubator (Thermo Forma), Centrifuge (Eppendorf -5415R); Nikon fluorescence micro-scope (E600).

2.2 Cell culture

The Hela cell line was purchased from the Chinese center for cell culture collection located in Wuhan University of China. Hela cells were cultured in a RPMI 1640 solution supplemented with 10% fetal calf serum in a humidified incubator with an atmosphere of 5% CO_2 in air at 37°C.

2.3 Preparation of MnO_2 particles suspensions

Nano-MnO_2 particles with the diameter of 20~100nm and Nor-MnO_2 particles with the diameter of 1~2μm were generously provided by the Center for Material Science and Chemical Engineering College of China University of Geosciences. Different contents of suspension (1, 2, 4mg·mL^{-1}) were prepared by saline solution, and then autoclaved at 121°C for 30 minutes. Before the peritoneal injection, all the suspension must be sonificated for 40 minutes.

2.4 Exposure of cells to MnO_2 particles suspensions

Hela cells were plated in 24-well plates, and after that cells were cultured for 12h at 37°C in CO_2
to allow cell attachment. Nano-MnO$_2$ particles suspensions and Nor-MnO$_2$ particles suspensions were then added respectively to the final concentration of 100, 200 and 400 $\mu$g·mL$^{-1}$. At the same time, the MnO$_2$-free medium was added to the cells for control group. The cells were recultured for 12h in the dark at 37°C, the MnO$_2$ solution was removed, and the cells were washed twice with PBS (pH = 7.2). Adjust the cell density to $10^4$–$10^6$·mL$^{-1}$.

2.5 Comet assay (SCGE)

The protocol was performed according to Tice et al. (2000), with some modifications. Briefly, Hela cells were cast into miniature agarose gels on microscope slides and lysed in situ to remove DNA associated proteins and allow the compacted DNA to relax in lysis buffer (2.5mM NaCl, 100mM EDTA, 10mM Tris-HCl (pH=10), 1% Triton X-100, and 10% DMSO). After lysis at 4°C for 2h, proteinase K was added to the lysis solution (final concentration 10mg·mL$^{-1}$) and additional lysis was performed at 37°C for 2h. Following cell lysis, all slides were washed through three changes of deionized water at 20min intervals to remove salt and detergent from the microgels. Slides were placed in a horizontal electrophoresis unit and were allowed to equilibrate for 20min with TBE buffer (300mM NaOH, 1mM EDTA, pH=13) before electrophoresis (17V, 240mA) for 20min. When electrophoresis was complete the slides were rinsed with water, air-dried, and stored protected from light until analysis.

2.6 Data analysis

Origin 5.0 software was used for the t-test and applied to determine DNA damage, as shown by the migration of DNA fragments in the agarose gel. Tail Moment and Tail DNA% were estimated to indicate DNA migration. Overall, at least 50 cells per sample were analyzed by the use of fluorescent microscope (WH-2, Olympus) and image analysis software (CASP, from http://www.casp.of.pl). Statistical differences were considered significant at $p<0.05$.

3 Results

Fig.1 and Fig.2 showed the analyzed images after analysis by CASP software of control and Nano-MnO$_2$ exposure groups. From the images it can be seen that control cells showed tight and round DNA heads, and have no obvious tails. In contrast, Nano-MnO$_2$ exposed cells showed typical comet shapes. DNA heads are relatively brighter, while the tails are composed by DNA slides, and are darker compared with head.

![Fig.1 Comet images from the experiment](image1)

(A: the control group; B: the 400$\mu$g·mL$^{-1}$ Nano-MnO$_2$ group)

![Fig.2 The analysis with CASP on the images](image2)

(A: the control group; B: the 400$\mu$g·mL$^{-1}$ Nano-MnO$_2$ group)
The result of different concentration of Nano-MnO₂ and Nor-MnO₂ to the DNA damage was shown in Fig.3. Compared with the control group, every group treated by Nano-MnO₂ and Nor-MnO₂ has a significant increase in Tail DNA% and Tail Moment (p < 0.01). With the increase of the concentration, the DNA damage was significantly increased, showing an significant dose-effect relationship. And at the same concentration, the Tail DNA% and Tail Moment of Nano-MnO₂ group were significantly higher than those of the Nor-MnO₂ group (p < 0.01), which indicated that the oxidize damage to the DNA in Hela cells by Nano-MnO₂ was much higher than Nor-MnO₂.

Fig.3 Tail DNA% and Tail Moment induced by Nano-MnO₂ and Nor-MnO₂

(**: compared with the control group, p<0.01; ###: compared with Nor-MnO₂-treated groups, p<0.01)

4 Discussion

4.1 The possible mechanism of oxidize damage to the DNA in Hela cells by Nano-MnO₂

Nel et al. (2006) published a review in Science and indicated that the generation of ROS and oxidative stress were the main mechanism of most of the toxic effect which was induced by nanoparticles. The surface properties of Nano-MnO₂ can lead to the interaction of electron donor or acceptor active sites (chemically or physically activated) with molecular oxygen (O₂). Electron capture would result in the formation of the superoxide radical (O₂⁻), which through dismutation or Fenton chemistry can generate additional ROS. ROS is the reduction metabolic of the oxidative stress reaction of many cells, including free radical O₂⁻, NO and unfree radical H₂O₂, ONOO⁻. If the cells can generate one kind of ROS, it then can generate other kind of ROS by chain reaction. A suitable dose of ROS can modulate physical function, while excess ROS can increase the oxidize pressure in cells and bodies, and can attack nucleotide and lead to DNA single strand breakage (Wu et al., 2006). Papageorius et al. (2007) checked the effect of nano-cobalt-chromium alloy and micro-cobalt-chromium alloy to the human fibroblasts cell by SCGE, and found that nano-cobalt-chromium alloy could induce more ROS and damage the DNA significantly. Our data showed that exposure to the Nano-MnO₂ 12h can induce the DNA damage in Hela cells significantly, and DNA damage and the toxicants concentrations has a significant dose-dependent relationship. So, we suppose that Nano-MnO₂ can also induce DNA damage by generating excess ROS. In spite of this, the molecule mechanism of the Nano-MnO₂ to the DNA damage is still not known and further research need to be done.

4.2 Relationship between particles’ size and toxic effects

In present experiment, Nano-MnO₂ induced significantly more DNA damage in Hela cells than that of Nor-MnO₂, indicating the direct relationship between MnO₂ particles’ size and its toxic effects. That is, under certain extents, the smaller the size is, the more serious DNA damage effect is. Other studies showed similar results. Ying et al. (2006) showed that Nano-SiO₂ induced more serious lung damage of rats than that of Nor-SiO₂. Wang et al.
summarized available studies about biological effects of Nano-TiO₂ and found that Nano-TiO₂ particles had more serious damages on lungs compared with Nor-TiO₂. It is reported that the diameter-toxic effect may be due to the size dependent effects of nanomaterials. As an important characteristic of nanomaterials, size dependent effects mean the changes in macroscopical physical characters when the particle size becomes smaller. When the particle size becomes smaller, the atoms at the surface of the nanomaterials become quite reactive and unstable, and are easier to react with other atoms, which may account for its improved biological toxic effects.

In this experiment, equivalent quality of particles of the same concentrations of Nano-MnO₂ and Nor-MnO₂ was applied to cells. The results suggested that with the same quality of particles, Nano-MnO₂ may be more active and thus has greater toxic effects.

During last 10 years nanomaterial is used more and more widely. However, since the absence of research models and evaluation methods, the biological safety of nanomaterials remains unknown. In this experiment, Nano-MnO₂ showed more serious DNA damage effect compared with Nor-MnO₂, suggesting that Nano-MnO₂ is of size-dependent toxicity. Our data about the size-dependent toxicity of Nano-MnO₂ are consistent with other reports, and is therefore credible and provides basis for the safety guideline of the use of Nano-MnO₂. Meanwhile, as a common detection method for DNA damage, single-cell gel electrophoresis can be successfully applied in DNA damage effect of ultrafine particles, and may provide references for other similar researches.

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