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Oxidative stress and apoptosis induced by nanosized titanium dioxide in PC12 cells

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ABSTRACT

The nanosized titanium dioxide (nano-TiO_2) is produced abundantly and used widely in the chemical, electrical/electronic and energy industries because of its special photovoltaic and photocatalytic activities. Past reports have shown that the nano-TiO_2 can enter into the human body through different routes such as inhalation, ingestion, dermal penetration and injection. The effects of nano-TiO_2 on different organs are currently being investigated and the concerns on its large scale applications such as sunscreen, etc. indeed become more interesting for us to investigate and to find the possible right answers for right doses for a safer application. In this research, the cytotoxicity of the nano-TiO_2 was investigated in PC12 cells, a cell line used as a model in vitro for the brain neurons research. While the PC12 cells were treated with different concentrations of nano-TiO_2 (1, 10, 50 and 100 μg/ml), the viability of cells was significantly decreased in the periods of 6, 12, 24 and 48 h, showing a significant dose effect and time-dependent manner. Meanwhile, the flow cytometric assay gave indication that the nano-TiO_2 induced intracellular accumulation of reactive oxygen species (ROS) and the apoptosis of PC12 cells with the increasing concentration of nano-TiO_2. Interestingly, pretreatment of N-(mercaptopropionyl)-glycine (N-MPG), known as a type of ROS scavenger formulations, could somehow inhibit PC12 apoptosis induced by the nano-TiO_2. These results might have revealed a key mechanism in PC12 apoptosis under the effect of the nano-TiO_2 solutions.

1. Introduction

There are increasingly concerns about development and production route of nanomaterials and nanotechnology, as well as their wide ranges of applications. The new physical and chemical properties of novel engineered nanoparticle make them extremely attractive for use in the applications such as medical science (Emerich and Thanos, 2003), drug applications, agricultural, and defense industries (Long et al., 2007). Despite the wide ranges of applications, there is a serious lack of information on the impact of the nanoparticles on human health and the environment (Braydich-Stolle et al., 2005).

The nano-TiO_2, has now been produced in a large industrial scale and used widely due to its high physical stability, anticorrosion and photocatalysis (Wang et al., 2007; Sun et al., 2004; Gelis et al., 2003). Therefore, potential widespread exposure may occur during both manufacturing and end users (Wang et al., 2007). As an ultrafine sized material, the nano-TiO_2 can enter the human body through different routes such as inhalation (respiratory tract), ingestion (gastrointestinal tract), dermal penetration (skin), and injection (blood circulation) (Jin et al., 2008; Oberdorster et al., 2005). The extensive uses in medical research and industrial applications highlight that there are many routes for the nano-TiO_2 to potentially enter into human bodies. Moreover, there is also a considerable risk for exposure to livestock, and eco-relevant species (Long et al., 2007). In the past 10 years, it has also been reported that the nano-TiO_2, once inhaled, could cause inflammation, fibrosis, pulmonary damage (Afaq et al., 1998; Rahman et al., 2002). Amezaga-Madrid et al. reported that photocatalytic TiO_2 thin films can produce inhibition to bacterial in the range of 32–72%, to *Pseudomonas aeruginosa*, which indicating the possible inhibition of cell growth by the nano-TiO_2 (Amezaga-Madrid et al., 2003). Work done in relations to nanomaterials to intracellular calcium concentration showed a rapid increase when the malignant cells were treated with the nano-TiO_2, indicating the damage of the plasma membrane and followed the entrance of extracellular Ca^{2+} (Sakai et al., 1994).

PC12 cell is a cell line derived from a rat adrenal medulla pheochromocytoma. Differentiated PC12 cells induced by nerve growth factor (NGF) have the typical characteristic of the neurons in the form and function, and therefore are widely used

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as a model in vitro for the neuron research, such as the apoptosis and the differentiation of neuron (Rukenstein et al., 1991; Green, 1978; Xiao et al., 2008; Ishima et al., 2008). The viability of the cells was observed by a 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays. The oxidative stress induced by the nano-TiO_2 and their mechanism was studied in relation to the generation of reactive oxygen species (ROS).

2. Materials and methods

2.1. Materials

RPMI 1640 cell culture medium was purchased from GIBCO Invitrogen. MTT, NGF, horse serum and fetal bovine serum (FBS) and N-(2-mercaptopropionyl)-glycine (N-MPG) were all purchased from Sigma Chemical Co., St. Louis, MO, USA. ROS testing kit was purchased from Genmed Scientifics Inc., USA. Annexin V-FITC propidium iodide (PI) apoptosis detection kit was from Bipec Biopharma Corporation, USA. Plastic culture microplates and flasks used in the experiment were supplied by Corning Incorporated (Costar, Corning, NY, USA).

2.2. Dispersion and characterization of the TiO_2 nanoparticles

Commercial titanium dioxide nanoparticles (P-25 type, 21 nm in average size) were provided by Research Institute of Science and Technology (RSTI), University of Hertfordshire, England. The material is photoactive, largely anatase form of TiO_2. The sizes of particles were tested using Transmission Electron Microscopy (TEM, Tecnai G2 20 S-TWIN, FEI, USA). And the average aggregate or agglomerate size of the nano-TiO_2 was measured by dynamic light scattering (DLS). Here, the nano-TiO_2 was suspended in RPMI 1640 cell culture medium and dispersed by an ultrasonic vibrator for 10 min, and then the suspension was characterized by the DLS using a Zeta PALS + BI-90 Plus (Brookhaven Instruments Corp., USA) at a wavelength of 659 nm. The scattering angle was fixed at 90°.

2.3. Cell culture and nanoparticle suspension preparation

PC12 rat pheochromocytoma cells were obtained from Institute of Basic Medical Sciences Chinese Academy of Medical Sciences. The cells were kept on plastic culture microplates with RPMI 1640 (pH 7.4) supplemented with 5% fetal bovine serum and 10% horse serum. In the presence of nerve growth factor (NGF), PC12 cells will differentiate into sympathetic-like neurons, the medium was then replaced to serum-free RPMI 1640 supplemented with 50 ng/ml NGF. The cells were cultured with the medium (with NGF) for 7 days, including changes every other day until the neurites were removed. Then the cells were cultured with the medium contain 10% FBS. All medium included 100 U/ml penicillin and 100 U/ml streptomycin. Cultures were propagated at 37 °C in a humidified atmosphere of 5% CO_2.

The suspension of titanium dioxide nanoparticles was prepared using the culture media and dispersed for 10 min by using a sonicator (Branson Inc., Danbury, CT, USA) to prevent aggregation. From the suspension, different final concentrations of the nano-TiO_2 were prepared in cell growth medium. The cells were then treated with various concentrations of the nanoparticle suspension.

2.4. Cell viability assay

The cell viability was assessed by using the MTT assay, which was based on the reduction of the dye MTT to formazan crystals, an insoluble intracellular blue product, by cellular dehydrogenases (Denizot and Lang, 1986). Cells were seeded on 96-well plates with 1×10^4 cells in 200 μl medium per well and cultured 24 h for stabilization. Then the freshly prepared nanoparticle suspension was dispersed in the cell culture medium and diluted to appropriate concentrations (1, 10, 50 and 100 μg/ml). Cells were cultured in the medium containing different concentrations of the nano-TiO_2 for the periods 6, 12, 24 and 48 h. Culture medium without the nano-TiO_2 served as the control in each experiment. At the end of the exposure, 20 μl MTT was added to each well into a final concentration of 2 mg/ml and afterwards the cells were cultured for 4 h at 37 °C. The medium was then removed carefully and 150 μl DMSO was added in and mixed with the cells thoroughly until formazan crystals were dissolved completely. This mixture was measured in an ELISA reader (Elx 800, Bio-TEK, USA) with a wavelength of 570 nm. The cell viability was expressed as a percentage of the viability of the control culture. Meanwhile, the concentration of the nano-TiO_2 used in assays of ROS and apoptosis were based on the results of the MTT test.

2.5. Measurement of ROS

The generation of ROS for the cells was evaluated by a fluorometry assay using intracellular oxidation of DCFH-DA. The cells in logarithmic growth phase were incubated in a 6-well plates for 24 h for stabilization, then the medium was replaced with medium containing different concentrations (0, 10, 50 and 100 μg/ml) of nano-TiO_2 for 24 h. After exposure, the cells were washed with phosphate-buffered saline (PBS), then they were resuspended at a concentration of 1×10^6 cells/ml and were stained by the staining solution for 20 min, the cells were detected and analyzed by flow cytometry.

2.6. Detection of apoptotic cells with flow cytometry

Apoptosis was assessed by annexin V-FITC and PI staining followed by analysis with flow cytometry (Beckman-Coulter, USA). The methodology followed the procedures as described in the annexin V-FITC/PI detection kit. The cultured cells were exposed to the suspension of the nano-TiO_2 with the concentration of 10 and 50 μg/ml for 24 h. Meanwhile, the cells and nano-TiO_2 (50 μg/ml) mixture were pre-treated with N-(2-mercaptopropionyl)-glycine (N-MPG) and followed incubation. Eventually, the cells were resuspended in a 400 μl 1× binding buffer solution with a concentration of 1×10^6 cells/ml, and the cells were stained with 5 μl annexin V-FITC and 10 μl PI for 15 min at room temperature in the dark. Then the cell suspension was ready for the analysis by the flow cytometry.

2.7. The effect of N-(2-mercaptopropionyl)-glycine (N-MPG)

The effect of N-MPG, known as a kind of ROS scavenger (Mitsos et al., 1986), was detected by MTT assay. After stabilized on a 96-well plates, cells were pre-treated with N-MPG (300 μmol/L concentration) for 30 min. Following the procedures of standard method described above the nano-TiO_2 suspension was to the final concentration of 50 μg/ml and followed incubation for 24 h. Cells cultured without the nano-TiO_2 were served as the control.

2.8. Statistical analysis

The results were expressed as mean ± SEM. The results of the groups treated with the suspension of the nano-TiO_2 were compared to those of the non-treated control group and represented as the percentage of the control value. Data were evaluated using ANOVA followed by Dunnett's multiple comparison post hoc test. A P-value of less than 0.05 was considered to be statistically significant.

3. Results

3.1. Characterization of nano-TiO_2

The size of the nano-TiO_2 was distributed from 20 to 50 nm as shown in the images of Transmission Electron Microscopy (TEM) (Fig. 1a). The dynamic light scattering (DLS) observation showed that the particle size distribution had a wide range from 24 to 697 nm due to the aggregation or agglomeration, and the hydrodynamic diameter was 368.1 nm (Fig. 1b).

3.2. Cytotoxicity of the nano-TiO_2

The MTT assay was used to examine the cytotoxicity of the nano-TiO_2. PC12 cells were treated with medium containing different concentrations (1, 10, 50 and 100 μg/ml) of the nano-TiO_2, and viability was determined at 6, 12, 24 and 48 h after treatment. As shown in Fig. 2, cell viability was decreased when the concentration and time period increased. After 6 h incubation, cell viability was decreased, the viability of the cells incubated with the concentrations (1, 10 and 50 μg/ml) was not greatly altered, but only the concentration of 100 μg/ml showed significance. With time increasing, significant cytotoxicity of the nano-TiO_2 was observed at the concentrations of 10, 50 and 100 μg/ml. ANOVA analysis and Dunnett's test revealed that PC12 cells were inhibited both in a dose-dependent and time-dependent manner by the nano-TiO_2.

3.3. Measurement of ROS generation

To investigate whether the nano-TiO_2 stimulated ROS generation in PC12 cells, the intracellular ROS level was measured using the ROS test kit. DCFH-DA can passively enter the cell and react with the ROS to produce a fluorescent compound dichlorofluorescein (DCF). When the membrane is oxidized and damaged, the fluorescence will attenuate significantly. After the PC12 cells were exposed to the nano-TiO_2 with the different concentrations of 0, 10, 50 and 100 μg/ml for 24 h, the generation of the ROS was proved by the increased fluorescence intensity of oxidized DCF. As shown

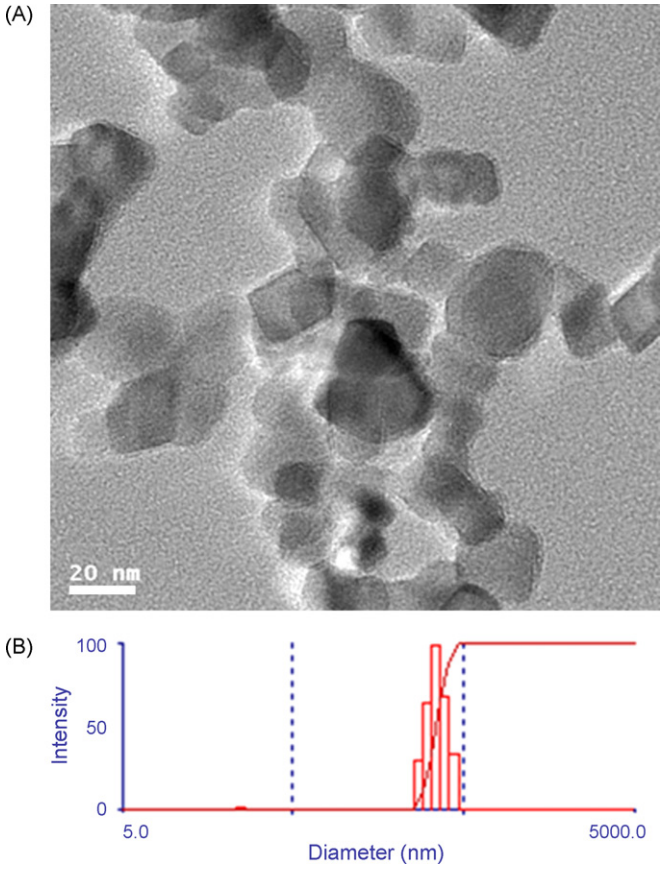

Fig. 1. Dispersion and characterization of the TiO_2 nanoparticles were characterized by TEM (a) and DLS (b). TEM images showed that the size of the nano-TiO_2 was distributed from 20 to 50 nm. And the DLS assay (b) stated that the particle size distribution had a wide range from 24 to 697 nm due to the aggregation, and the hydrodynamic diameter was 368.1 nm.

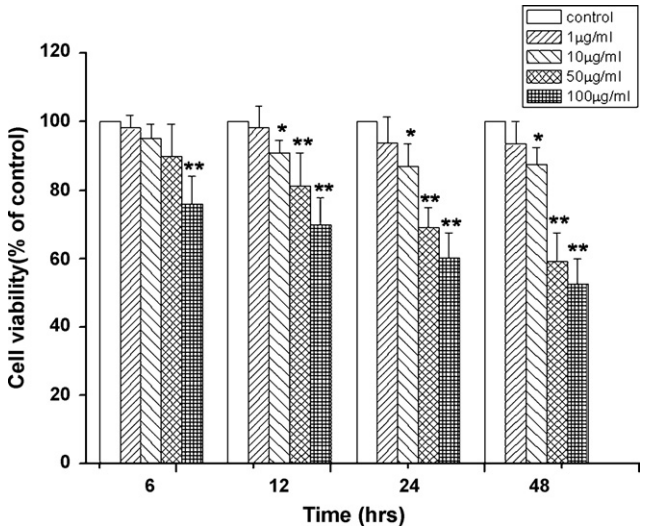

Fig. 2. The viability of PC12 cells treated with different concentrations of the nano-TiO_2. Cell viability was assessed by MTT assays and the results were presented as a percentage of control group viability. Cells were incubated with the nano-TiO_2 (1, 10, 50 and 100 μg/ml) for 6, 12, 24 and 48 h. The result showed that cell viability was greatly reduced in a dose-dependent and time-dependent manner after exposure to the nano-TiO_2. Results represent the means of three separate experiments, and error bars represent the standard error of the mean. (Statistics: one-way ANOVA with Dunnett's post hoc test. $^{*}P < 0.05$ compared with the control group, $^{**}P < 0.01$ compared with the control group.)

in Fig. 3, the ratio of DCF-positive cells was 3.72%, 24.45%, 51.49% and 82.37% at the concentration of 0, 10, 50 and 100 μg/ml, respectively. The results revealed that the level of ROS in PC12 cells, which treated with the nano-TiO_2 suspension, was increased in a positive proportion of dose-dependent.

3.4. The apoptosis of PC12 cells induced by the nano-TiO_2

The apoptosis of PC12 cells was tested by flow cytometry, as shown in Fig. 4, the apoptosis rate of PC12 cells was increased from 5.27% in control group to 11.34% and 23.47% after the cell exposure to the nano-TiO_2 (10 and 50 μg/ml) for 24 h, respectively. However, a pre-treatment to the cells using N-MPG (300 μmol/L) reduced the cellular apoptosis to 10.26%. The result indicated that the nano-TiO_2 induced apoptosis of PC12 cells in a dose-dependent manner, but pre-treated with the ROS scavenger can inhibit the nano-TiO_2 induced apoptosis in PC12 cells.

3.5. Viability of PC12 cells with the pre-treatment of N-MPG

Cell viability was tested by MTT assay. As shown in Fig. 5, the viability of PC12 cells cultured with the nano-TiO_2 (50 μg/ml) for 24 h was 42.42%. When pre-treated with ROS scavenger N-MPG, the viability of PC12 cells was significantly increased to 98.01%.

4. Discussion

Nanotechnology involves the creation and manipulation of materials at nanoscale to create products that exhibit novel properties (Hussain et al., 2005). Nanomaterials, which range from 1 to 100 nm, have been used to create unique nanosized devices possessing novel physical and chemical properties (Suzuki et al., 2007). Because of these special properties, nanomaterials are widely used in many fields. nano-TiO_2, a kind of nanomaterials, is widely used because of its unusual properties. Therefore the potential risk of nanoparticles to biological systems is needed to be investigated.

Several studies had reported that the nano-TiO_2 can cause the damage of different cells such as human lymphoblastoid cells (Wang et al., 2007), syrian hamster embryo fibroblasts (Rahman et al., 2002) and BEAS-2B cells (Park et al., 2008). The ultrafine TiO_2 particles could be incorporated into cellular membranes, and might be endocytosed from the extracellular fluid and made fused with lysosomes, then led to the damage and destruction of organelles (Jin et al., 2008).

In this study the cytotoxicity of the nano-TiO_2 was assessed in PC12 cells cultured with different nanoparticle concentrations. Firstly the characterization of the nano-TiO_2 was investigated as shown in Fig. 1. The TEM images showed that the nano-TiO_2 was with a diameter <50 nm (from 20 to 50 nm). This size range of anatase was considered to be more toxic than an equivalent sample of rutile (Sayes et al., 2006; Jin et al., 2008). Hussain et al. reported that the nano-TiO_2 induced inflammatory effects mediated through oxidative stress and the biological responses were related to the BET (Brunauer, Emmett and Teller) surface area of the nano-TiO_2 and the internalized amount (Hussain et al., 2009). A study in vivo showed the micro-scale of nano-TiO_2 (20 nm particle size) led to a greater pulmonary inflammatory response than that of large scale of nano-TiO_2 (250 nm) (Baqqs et al., 1997). The past research indicated that the different sizes of nanoparticles caused varying degrees of damage. In this study, we just studied the injury caused by the nano-TiO_2 with the size of 25 nm, and the influence induced by other size of nano-TiO_2 need further study. In this study, the viability of PC12 cells incubated with the nano-TiO_2 in the concentration range was investigated. The MTT test showed that cell viability was greatly reduced in a concentration-dependent and time-dependent manner (Fig. 2). Also, the detection

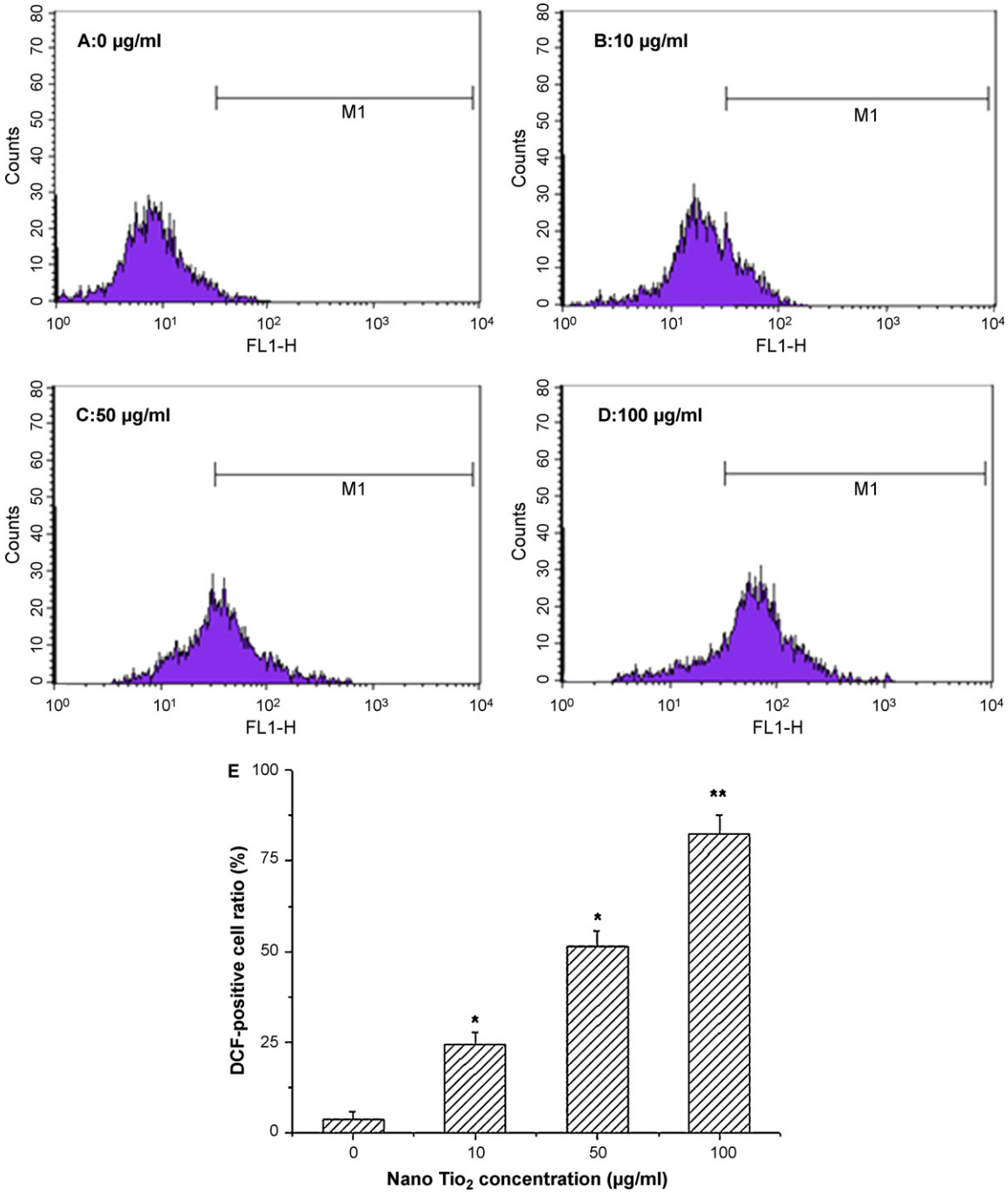

Fig. 3. Measurement of ROS generation in PC12 cells. The cells were cultured with the nano-TiO_2 at concentrations of 0 μg/ml (A), 10 μg/ml (B), 50 μg/ml (C), 100 μg/ml (D) for 24 h. The generation of ROS was measured by flow cytometry. The level of ROS was increased as a dose-dependent manner. The corresponding linear diagram of flow cytometry was shown (E). $n = 3$, mean ± SEM, *statistically significant difference compared with controls ($P < 0.05$), **$P < 0.01$.

of the cell apoptosis showed that the nano-TiO_2 induced the apoptosis of PC12 cells. ANOVA analysis demonstrated that there was a statistical significance between different groups (Fig. 4, $P < 0.05$). The results indicated that the nano-TiO_2 decreased the cell viability and induced an apoptosis of the cells.

The cell apoptosis usually occurs when there is a destruction of the internal environment. ROS is an important factor in the apoptotic process. Excessive generation of ROS induces mitochondrial membrane permeability and damages the respiratory chain to trigger the apoptotic process (Jezek and Hlavata, 2005; Valko et al., 2006; Park et al., 2008). It was reported that when the cells exposed to the nano-TiO_2, there was an increase in the antioxidant enzymes in rat alveolar macrophages, but the cells still showed an enhancement of lipid peroxidation and increased the rate of hydrogen peroxide generation, which suggested that the nano-TiO_2 may lead to the oxidative stress and the induction of antioxidant enzymes by the cell self-protection. This might not be sufficient enough to cope in against the toxic action of the nano-TiO_2 (Afaq et al., 1998).

In this study, we measured the ROS generation using fluorescence microscope in DCFH-DA-treated PC12 cells (Fig. 3). The result indicated that when the cells were incubated with the different concentrations of nano-TiO_2, oxidative stress was occurred in response to the treatment of the nano-TiO_2, and the contents

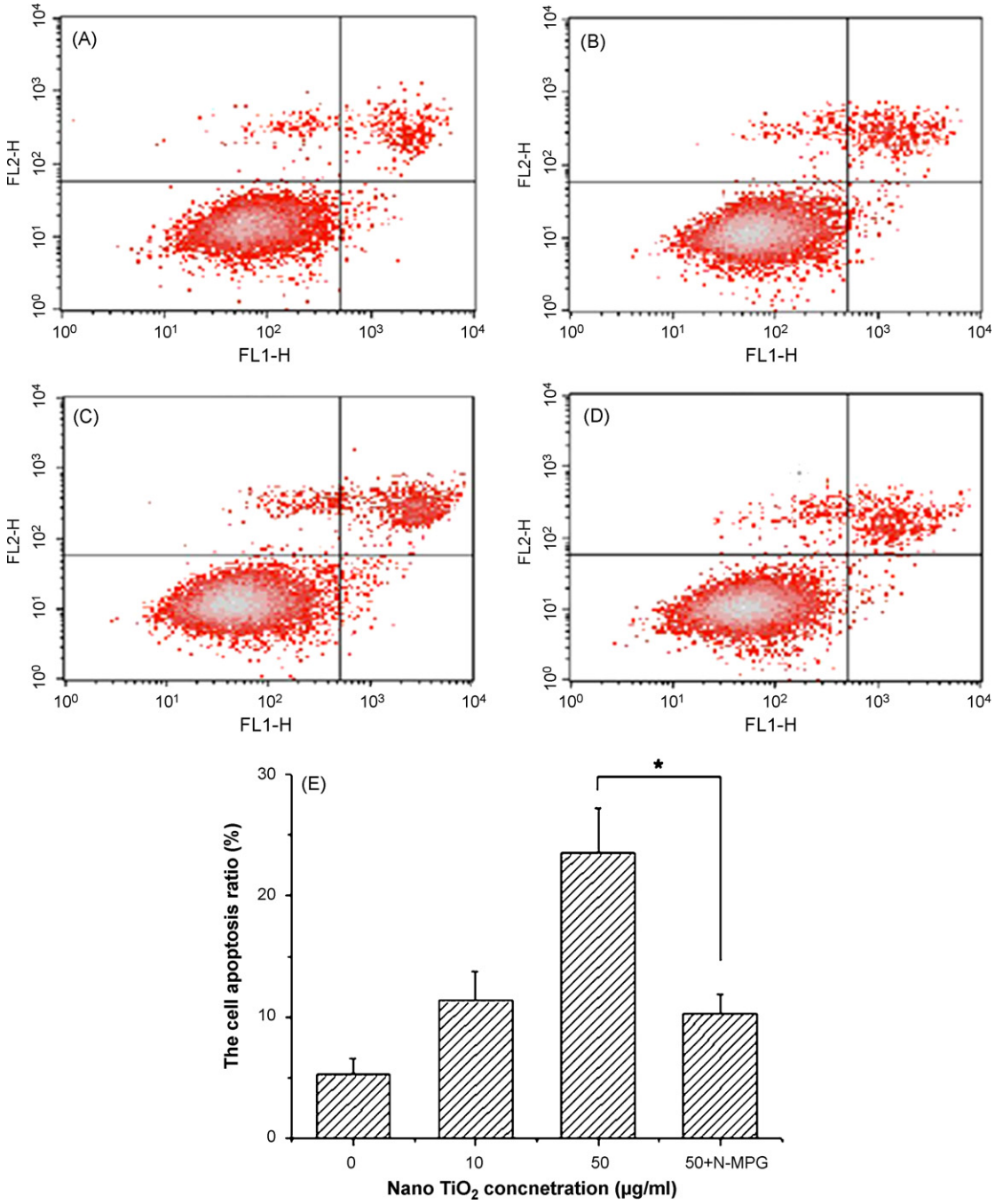

Fig. 4. The nano-TiO_2 induced apoptotic cell death in PC12 cells. The flow cytometry assay was carried out for detection of apoptotic cells. (A–C) The cells were treated with 0 μg/ml (A), 10 μg/ml (B), 50 μg/ml (C) of nano-TiO_2 for 24 h. (D) The cells incubated with the nano-TiO_2 (50 μg/ml) were pre-treated with N-MPG. (E) The corresponding linear diagram of flow cytometry was shown. $n = 3$, mean ± SEM, $^*P < 0.05$.

of the ROS was increased significantly ($P < 0.05$, $P < 0.01$). When pre-treated with N-MPG, a kind of ROS scavenger, there was a visible effect that the ROS scavenger can reduce the cell damage (Fig. 5, $P < 0.01$). These results demonstrated that the nano-TiO_2 might induce the apoptosis of PC12 cells by enhancement of intracellular ROS generation. Also the results showed that N-MPG could improve the cellular viability and reduce the damage caused by the nano-TiO_2.

Recent researches have demonstrated that oxidative stress and ROS generation play an important role in the pathogenesis of many neurological diseases, as the brain contains low cellular concentration of antioxidants and a large quantity of unsaturated fatty acids which are easy to be oxidized (Ray et al., 2001). Some studies indicated that nanoparticles can cross the blood–brain barrier (Lockman et al., 2004; Oberdorster et al., 2004) and enter (in low numbers) the central nervous system (CNS) of the exposed animals (Kreyling et al., 2002; Oberdorster et al., 2004). The study in our lab reported that the nanoparticles of CuO could affect the normal functions of the nervous system and may have potential danger to nervous system disease (Xu et al., 2009). Oberdorster et al. found that airborne solid ultrafine particles targeted CNS via the olfactory nerve (Oberdorster et al., 2004), and there were some other studies focused on the effect that nanoparticles made when existed in CNS (Zhao et al., 2009). The findings of this study are an important contribution to the researches on the neurotoxicity of the nanoTiO_2. Meanwhile, the results provide indirect

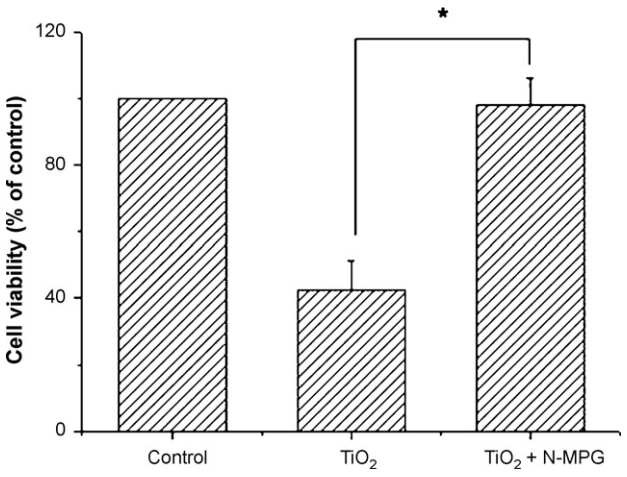

Fig. 5. Effect of N-MPG on the viability of PC12 cells incubated with the nano-TiO_2. Cells were pre-treated with N-MPG (300 μmol/l concentration) for 30 min, then the nano-TiO_2 suspension to the final concentration of 50 μg/ml and followed incubated for 24 h. Cells cultured without the nano-TiO_2 served as the control. Results represent the means of three separate experiments, and error bars represent the standard error of the mean, $^*P < 0.05$.

data for the application safety based on maximum dose in neuron cells.

In conclusion, the present study addressed potential mechanism of cellular apoptosis and revealed that nano-TiO_2 can induce a significant cytotoxicity in PC12 cells in a dose-dependent and time-dependent manner. Further studies are required to investigate the molecular mechanism of the cell apoptosis caused by nano-TiO_2.

Conflicts of interest

None declared.

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