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In vitro toxicity of iron oxide nanoparticle: Oxidative damages on Hep G2 cells

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ABSTRACT

During the past years many studies have been done highlighting the great need for a more thorough understanding of cell–iron oxide nanoparticle interactions. To improve our knowledge in this field, there is a great need for standardized protocols that would allow to comparing the cytotoxic potential of any Fe_2O_3-NP type with previously studied particles. Several approaches are reported that several parameters which are of great importance for Fe_2O_3 nanoparticle induced toxicity. Nanoparticles because of their very small size can pass through the cell membrane and can make oxidative damage in all parts of the cells such as mitochondria, membrane, DNA due to high surface area. This study focuses on acute cytotoxicity of reactive oxygen species and DNA damaging effects of mentioned nanoparticles. Results showed increase of the oxidative damage leads cells to the apoptosis, therefore reduced cell viability. It is interesting that all of the results are concentration and time dependent.

1. Introduction

Nanoparticles (NPs), which are defined as particles having at least one dimension of 100 nm or less, are used to produce novel materials with unique physicochemical properties (Gupta and Gupta, 2005). Nanoparticles because of their very small size have unique features such as high reactivity, high surface area, passing from the cell membrane, etc. Nanoparticles can pass through cell membrane easily and even pass through blood–brain barrier and blood–testes barrier (Mc Auliffe and Perry, 2007) so all of the body organs are affected by them (Yousefi Babadi et al., 2012).

Some nanoparticles such as iron, cobalt and nickel are known as magnetic nanoparticles because of their magnetic properties and stability (Jani et al., 1990; Lu et al., 2007). Iron oxide nanoparticles have widespread application for in vivo and in vitro research due to the physiochemical characteristics (Sun et al., 2008). Iron oxide nanoparticle has an enormous potential in the field of human imaging and early recognition of disease, with the use of specific nano-agents for molecular imaging in the context of Magnetic Resonance Imaging, ultrasound, optical imaging, and X-ray imaging. Magnetic nanoparticle also can be used widely in drug delivery, gene delivery and targeting (Indira and Lakshmi, 2010). For the above mentioned application magnetic nanoparticles should be biocompatible and biodegradable. There are growing concerns about the biological harm that this material can cause because of its widespread application. Usage of these particles expose human and animals to these materials, therefore investigation of nanoparticle role in cell growth and survival has importance (Zhu et al., 2009).

One of the toxic side effects of nanomaterials is oxidative damage caused by ROS production (Miura and Shinohara, 2009). One of the main reasons for this toxicity is high surface area to volume ratio (Buzea et al., 2007). Some of the in vitro and in vivo studies demonstrated oxidative damage by treatment of nanoparticle (Mohammadi Fartkhooni et al., 2013; Prodan et al., 2013). One of the main evidence for oxidative damage to the cell membrane is release of intracellular enzymes such as lactate dehydrogenase (LDH) out of cell and tissue respectively in in vivo and in vitro studies (Fotakis and Timbrell, 2006; Diamantino et al., 2001). Reduced glutathione (GSH) is one of the most important barrier against oxidative damage that is usually decreased by increasing ROS in living systems, therefore it is one of the oxidant representive factors in the body (Miura and Shinohara, 2009).

Nanoparticles between 10 and 100 nm size should be used for biological purpose because nanoparticles that are smaller than 10 nm are excreted by the kidneys. Particles larger than 200 nm do not pass through the cell membrane easily and they can be induced

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by immune system as a foreign thing therefore removed from the body (Gupta and Gupta, 2005). Thus, we used Fe_2O_3 nanoparticle with 50 nm size. Such a comprehensive study about the biological aspects of the iron oxide nanoparticle has not been done yet.

In this study we evaluated the toxic effects of Fe_2O_3 nanoparticle on human hepatoma cells (Hep G2). Liver is the primary detoxification organ, so hepatic cells continuously exposed to the toxicants xenobiotic agents. Hep G2 cells exposed to the different concentration of the Fe2O3-NP (25, 50, 75 and 100 mg/ml) for 12 and 24 h. We assessed cells viability potential by common MTT test. We also measured LDH, ROS and GSH concentration and DNA damage to evaluate oxidative damage of Fe_2O_3 on the Hep G2 cells.

2. Materials and methods

2.1. Chemicals

Roswell Park Memorial Institute (RPMI), penicillin, streptomycin, fetal bovine serum (FBS), 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), 2,7 dichlorofluorescin diacetate (DCFHDA), metaphosphoric acid (MPA), dimethyl sulfoxide (DMSO), o-phthaldialdehyde (OPT), and 5,5′-dithiobis (2-nitrobenzoic acid) were obtained from Sigma Chemical Company.

Nano ferric oxide (Fe_2O_3) particles prepared from Sigma–Aldrich Company. Nano-Fe_2O_3 is in crystalline phase with 50 nm size. Purification of nano Fe_2O_3 determined as 99.5% by ICP-MS. Toxicological assessment of manmade nanomaterials requires information about the route of exposure, as well as their complete physicochemical characterization of them in order to provide thorough information as summarized in Table 1.

2.2. Cell culture

Human hepatoma Hep G2 cells (that obtained from National Center for Cell Sciences, Pasteur Institute of Iran, Tehran) were grown in RPMI-1640 media supplemented with 2 mg/ml sodium bicarbonate, 10% (v/v) fetal bovine serum, 100 unit/ml penicillin, and 100 μg/ml streptomycin. The cells were cultured at 37 °C in a 5% CO_2 incubator. Hep G2 cells were treated with various concentrations of Fe_2O_3-NP and untreated cells used as control.

2.3. Fe_2O_3-NP treatment protocol

Before treatment Fe_2O_3-NP was dispersed by sonication (10 min, 750 W and 20 kHz) in suitable buffer. Cell culture exposed to the prepaid nanoparticle solution at 25, 50, 75, and 100 μg/mL concentrations for 12 h and 24 h. Dose range of nanoparticle selected based on the results of previous study done (data not shown) with different doses of nanoparticles. Exposure of cells was performed with 80% confluence of cell in 25 cm^2 flasks and 24-well plates in a humidified atmosphere at 37 °C and 5% CO_2. Cells free of Fe_2O_3-NP were used as control cells throughout each assay.

Table 1
Physical parameters of nano Fe_2O_3 used in present study. These features are more important than chemical and biological properties of Fe_2O_3 nanoparticle.

Color	Brown
Morphology	Spherical
Crystalline phase	Gamma
Specific surface area	50–245 m^2/g
Size	50 nm
Purity	99.5%

2.4. MTT assay

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 (Mohseni Kouchesfehani1 et al., 2013). 5 × 103 cells were incubated in 96-well plates with 1 × 104 cells in 50 μl medium per well. Cells stabilized by 2 h of culturing. The cells were cultured in the medium containing different concentrations of the Fe_2O_3-NP for 12 and 24 h time periods. Culture medium without the Fe_2O_3-NP considered as the control in each experiment. At the end of the exposure, 20 μl MTT (5 mg/mL dissolved in PBS) was added to each well to a final concentration of 22 mg/ml and then the cells were cultured for 4 h at 37 °C. The medium was then removed carefully and 200 μl DMSO was added in and mixed with the cells thoroughly until formazan crystals were completely dissolved. This mixture was measured in an ELISA reader with a wave length of 570 nm. Cell survival rate was calculated as a viability percentage of the control culture.

2.5. LDH assay

The level of lactate dehydrogenase (LDH) released from human hepatoma cells (as a damaging effect of nanoparticle exposure) was measured to evaluate the cytotoxicity of Fe_2O_3-NP. Hep G2 cells were treated with 25, 50, 75 and 100 μg/ml of Fe_2O_3-NP for 12 h and 24 h. The cells harvested by centrifuge (2000 rpm, 5 min) and supernatant separated. The culture supernatants were transferred to clean flat-bottom suitable plate for enzymatic assay. The activity of LDH in the supernatants was determined using LDH detection kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's guideline. All samples were assayed in duplicates for LDH content by a microplate spectrophotometer (Thermo MK3, MA, USA).

2.6. ROS concentration measurement

The intracellular concentrations of ROS were determined by measuring the oxidative conversion of DCFH-DA to dichlorofluorescin (DCFH) as a fluorescent compound (Wan et al., 1993). Briefly, Hep G2 were cultured in 24-well plates for 12 h, then treated with 25, 50, 75 and 100 μg/ml of Fe_2O_3-NP for 12 and 24 h, incubated with DCF diacetate in culture medium for 15 min, and washed with cold phosphate buffer solution three times. The measurement of green fluorescence (oxidized DCFH) using a microplate fluorometer (LB 941, Berthold Technologies, Bad Wildbad, Germany) with fluorescence intensity (excitation and emission) was done in 488 and 530 nm, respectively). The total protein concentration was evaluated using BCA protein assay kits (Pierce, IL, USA). The cell-free wells containing only buffer contain Fe_2O_3-NP and DCFH were used to assess nonspecific particle-induced fluorescence. Fluorescence was reported as percentage compared with untreated control cells.

2.7. Measurement of intracellular reduced glutathione (GSH) level

Cellular levels of GSH were measured using Hissin and Hilf method (Hissin and Hilf, 1976). The method is based on a reaction between GSH and o-phthaldialdehyde (OPT) which causes fluorescence. Thus GSH concentration in a sample solution can be determined by observing the fluorescence at 360 nm excitation and 420 nm emissions. Hep G2 cells were seeded into a 25 cm^2 flask at a density of 1.0×10^5 cells. After 12 and 24 h exposure to Fe_2O_3-NP, the cells were harvested and pelleted by centrifugation at 5000 rpm for 5 min, then washed in PBS. The cells were homogenized in 200 μL of phosphate–EDTA buffer pH 8.0 and 80 μL of 20% metaphosphoric acid. The cell homogenate was centrifuged at 16,000 rpm at 4 °C for 30 min. The assay was performed by taking

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