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Research Paper

Cytotoxic Effects of Iron Oxide Nanoparticles on Hep G2 Cells

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Abstract

Iron oxide nanoparticles (Fe_2O_3-NPs) are being used in an increasing number of fields such as molecular imaging in the context of Magnetic Resonance Imaging, ultrasound, optical imaging, and X-ray imaging. This widespread application of different sized of Fe_2O_3-NP in the biomedical field raises concerns over their increasing expose to tissues and organs of the human and animals. This study investigated molecular and cytotoxic effects of Fe_2O_3-NP (20 nm) on Hep G2 cells (human liver cancer cell line). Nanoparticles are containing high surface area to volume ratio that converted them to reactive molecules; therefore we evaluated oxidative stress biomarkers by standard biochemical methods. Results showed cells treated by Fe_2O_3-NP produced high concentration of reactive oxygen species (ROS). Assessment of ROS content in different concentration of Fe_2O_3-NP approved increased ROS level directly accompanied by doubled nanoparticle does. ROS overproduction is accompanied by lactate dehydrogenase enzyme (LDH) leakage from the cells revealed to membrane damages in the presence of toxicant nanoparticle. Mentioned molecular effects increase cell death in does dependent manner that evaluated by MTT assay. Possibly due to small size of nanoparticle which allows fast and easy entry into the cells and increased ROS overproduction attack to the membrane and intracellular organelles that finally lead to cell death.

Keywords: Nanoparticles; Reactive Oxygen Species; Lactate Dehydrogenase Leakage; Membrane Injury; Apoptosis

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1. Introduction

Nanoparticles (NPs) with unique physicochemical properties are described as particles having one or more dimensions of the order of 100 nm or less [1]. They have special features such as high reactivity; high surface area to volume ratio, penetrating through the cell membrane and etc due to very small size. Nanoparticles can pass through cell membrane easily and even pass through blood-brain barrier and blood-testes barrier [2], so all of the body organs are affected by them [3].

Nanoparticles containing magnetic elements such as iron, cobalt and nickel are known as magnetic nanoparticles [4, 5]. Fe_2O_3-NPs have widespread application for invivo and invitro research due to the physiochemical characteristics and stability [6]. Magnetic Resonance Imaging (MRI), ultrasound, optical imaging, and X-ray imaging are main molecular imaging methods in early recognition of disease mainly used from magnetic nanoparticles specially Fe_2O_3-NP with small size. Magnetic nanoparticle also could be applicable in drug delivery, gene delivery and targeting according to previous results [7]. Nanoparticle should be biocompatible and biodegradable for the above mentioned usage. There are growing concerns about the biological harm that this material can cause because of its general application and high

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reactivity. Widespread request of these particles exposes human and animals to these materials, therefore investigation of nanoparticle role in cell growth and survival are important and classified in nano-toxicology field [8].

According to previous studies and nano-materials feature, oxidative stress is one of the main toxic side effects of nanoparticles that enhance reactive oxygen species (ROS) molecules in living systems [9]. The main reason that causes this type of toxicity is high surface area to volume ratio or high reactive surface [10]. Some of the invitro and invivo studies demonstrated various oxidative damage in the presence of different type of nanoparticles [11,12]. One of the toxic effects is oxidative damages to cell membrane that cause the release of intracellular enzymes such as lactate dehydrogenase (LDH) out of cell and tissue respectively in invivo and invitro studies [13, 14]. Nanoparticles that are smaller than 10 nm are expelled by the kidneys therefore particles between 10-100 nm size are used for biological purpose. Note that particles larger than 200 nm don't pass through the cell membrane easily and stimulate immune system as a foreign agent so will be removed from the body soon [15]. Thus, we used Fe_2O_3 nanoparticle with 20 nm size. Such a comprehensive studies about the biological effects of the Fe_2O_3-NP are rare. The liver is primary organ that deactivates toxic materials, so hepatic cells continuously exposed to the toxicants xenobiotic. In this study we evaluated the toxic effects of Fe_2O_3 nanoparticle on human hepatoma cells (Hep G2). Hep G2 cells were exposed to the different concentration of the Fe_2O_3-NP (25, 50, 75 and 100 mg/ml) for 12 and 24 hours. Cell viability potential was evaluated by common MTT test. ROS content, LDH leakage and DNA damage were assessed by standard biochemical methods and immunological tests.

2. Material and Method

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. 20 nm sized nano-ferric oxide (Fe_2O_3) particles prepared from Sigma-Aldrich Company.

2.2. Experimental design

Human hepatoma cells or Hep G2 (that obtained from National Center for Cell Sciences, Pasteur Institute of Iran, Tehran) were cultured 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 were used as control. Before treatment, Fe_2O_3-NP was dispersed by sonication (10 min, 750 W and 20 kHz) in suitable buffer. Cells exposed to the nanoparticle at 25, 50, 75, and 100 µg/ml concentrations for 12 h and 24 h. dose range of nanoparticle selected based on our previous studies. 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.

2.3. Cell viability assay

Cell viability was assessed by using the MTT assay, which was based on the reduction of MTT to formazan crystals, an insoluble intracellular blue product, by cellular dehydrogenases [16]. 5 × 105 cells were incubated in 96-well plates with 1×104 cells in 50µl medium per well. At the end of the experiment, 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 4h 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.

2.4. LDH assay

Released lactate dehydrogenase (LDH) from Hep G2 cells to the medium was measured to evaluate the cytotoxicity of Fe_2O_3-NP. Cells were treated with Fe_2O_3-NP at specific time duration. Control and nanoparticle received Hep G2 cells were harvested by centrifuge (2,000 rpm, 5 min) and amount of LDH enzyme was detected in supernatant by detection kit (Nanjing Jiancheng Bioengineering Institute, Nanjing, China) according to the manufacturer's guideline. All of the experiments were performed in triplicate.

2.5. 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 [17]. Briefly, cultured Hep G2 cells were exposed to Fe_2O_3-NP and then 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) was done using a microplate fluorometer (LB 941, Berthold

Technologies, Bad Wildbad, Germany) with fluorescence intensity (excitation and emission were done in 488 and 530 nm respectively). The total protein concentration was evaluated using Bradford method. 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.

2.6. DNA damaging assay

Damaging to DNA in control and nanoparticle received cells was detected using γ-H2AX, a phosphorylated form of H2AX, which bound to the sites of DNA double-strand breaks [18]. After treatment of HepG2 cells by nanoparticle during experimental duration, cells were centrifuged at 300 g for 4 min at room temperature. Then cells were washed with PBS buffer and fixed with 2 % paraformaldehyde in PBS for 30 min. The cells were blocked with bovine serum albumin (BSA) (3 % in PBS) for 30 min. After blocking, cells incubated with anti-γ-H2AX antibody (Millipore, Billerica, MA) (100-fold dilution in PBS and 1 % BSA) for 1 h, and then incubated with Alexa Fluor 488 goat anti-mouse IgG antibody (Invitrogen) at a 500-fold dilution for 1 h, and fixed with a DAPI (40,6-diamidino-2-phenylindole)- containing mount solution. A double-strand break in genomic DNA detected by fluorescence was observed by confocal microscopy.

2.7. Statistical evaluation

Three independent replicates were done in each experiment and data were expressed as mean±standard deviation (SD). The data were subjected to statistical analysis by one-way analysis of variance (ANOVA) followed by Dunnett's method for multiple comparisons. A value of $p<0.05$ was considered significant.

3. Results

3.1. Fe2O3–NP imposed cytotoxic effects according to exposing time and concentration

Cytotoxicity of Fe_2O_3-NP evaluated by MTT assay and LDH leakage assay on the Hep G2 cells [12, 16, 19, 20]. Cell viability that examined by MTT assay showed cell death increased by treating with Fe_2O_3–NP in a concentration dependent manner. Increased exposing time causes more significant reduction in cell viability. Consequence of nanoparticle exposure has toxic and negative effects on the cell survival such as other nano size materials [16, 19, 20]. Viability of Hep G2 decreased to 12 % by giving harsh dose (100 mg/ml for 24 h) and reduced to 20 % by adding the nanoparticle in 100 mg/ml concentration for 12 h (Figure 1A).

LDH is an intracellular enzyme that could penetrate to the medium (out of cells) in an unhealthy condition such as loss of cell membrane integrity [21]. Therefor measurement of LDH activity in cell culture is a best indicator for cytotoxicity according to previous studies [13, 14, 19]. Compared with the unexposed control cells, level of the released LDH from the cells significantly increased after 12 or 24 h of exposing to 25, 50, 75 and 100 µg/ml of Fe_2O_3-NP in a concentration and time dependent manner (Figure 1B). Figure 1B indicate amount of released LDH from the cell increased to more than 15 folds in treating by 100 µg/ml Fe_2O_3-NP for 24 h, so damaging effects of nanoparticle is very harsh in this condition.

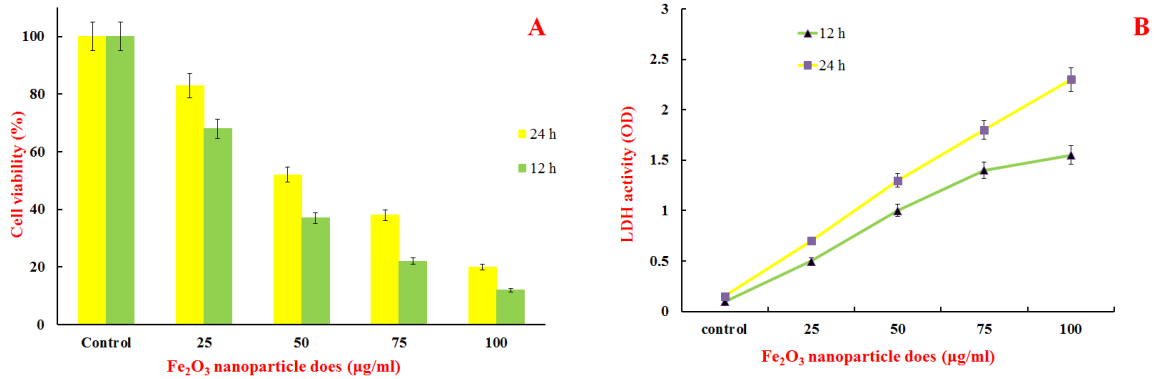

Figure 1. Nanoparticle toxicity assay. A: Measurement of cell viability by MTT assay. % Cell survival reduced in the presence of Fe_2O_3-NP concentration and time dependently. B: LDH assay in medium showed leaking of enzyme increased in the presence of nanoparticle because of membrane damages. Membrane damages enhance in 100 µgml of Fe_2O_3-NP.

3.2. Intracellular ROS content increased in the presence of nanoparticle

Reactive Oxygen Species (ROS) are chemically reactive molecules containing oxygen such as oxygen ions, super oxide and peroxides that present in the cells with very low concentration but harmful stress can increase them and damage to the cell by these reagents [22]. Therefore, evaluation of the ROS content in living system will give useful information about the oxidative condition of cells [19, 20. 23]. The concentration of ROS molecules that imposed by Fe_2O_3-NP was measured by DCF fluorescence intensity in Hep G2 cells. As shown in figure 2, ROS content of cells increased in a concentration and time dependent manner. The high level of ROS content observed in cells exposed for 24 h at 100 µg/ml of Fe_2O_3-NP.

3.3. Nanoparticle exposing causes double stand break in Hep G2 cells

Measurement of phosphorylated H2AX that bind to double strand breakages in the DNA is one of the best methods for evaluating of the DNA damage [24]. Figure 3 compared fluorescence intensity of cells that treated by 100 µg/ml nanoparticle for 12 and 24 h and control cells. Fluorescence intensity in cells that treated with nanoparticle during 24 h is significantly more than 12 h exposed cells that confirmed more DNA damages. Lack of any fluorescence intensity in the control indicates there wasn't any DNA damaging agent in the cell culture medium that was used in this study.

4. Discussion

Specific physicochemical features of nanoparticles such as size, shape and high surface area to volume ratio make them more applicable in biology and medicine. These materials could be speared in all of the body organs rapidly after injection by circulation and also could penetrate through cell membrane and affect cellular organelles [2, 6, 7, 10], therefore all of the body cells are exposed to these materials. Most of the previous studies demonstrated the main involved molecular mechanism in nanotoxicity is oxidative stress resulting from the ROS generation (9). A wide range of nanomaterials have been shown to create ROS both in vitro and in vivo conditions [11, 12].

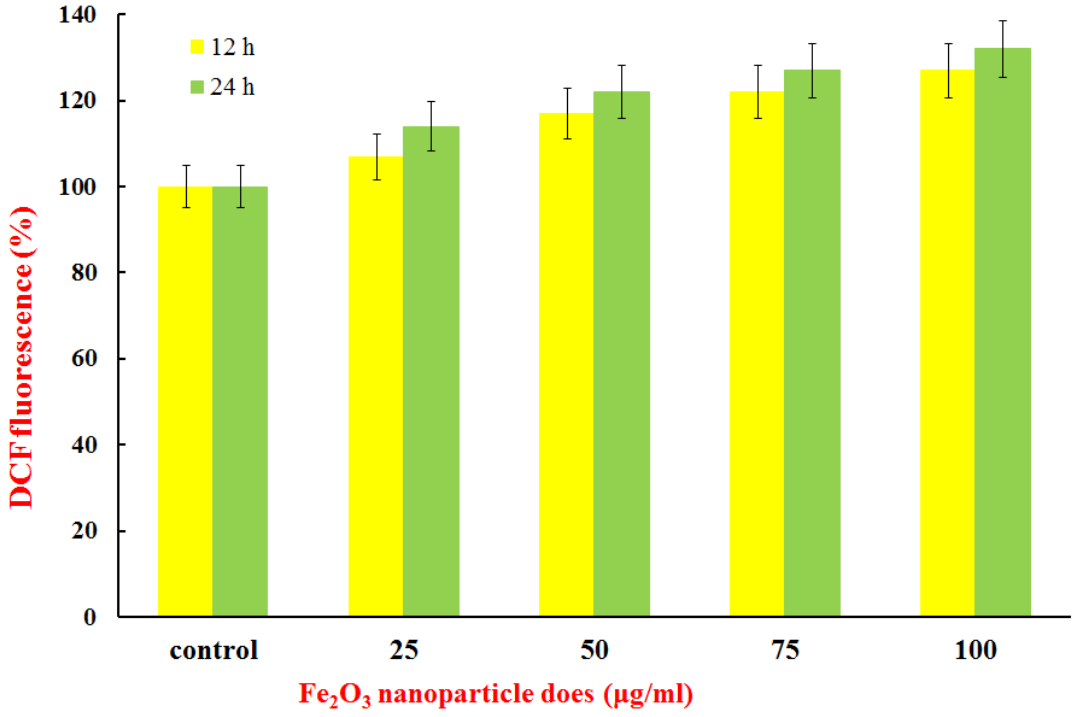

Figure 2. Oxidative stress assessment. ROS content of Hep G2 cells increased by Fe_2O_3-NP treatment in a concentration and time dependent manner.

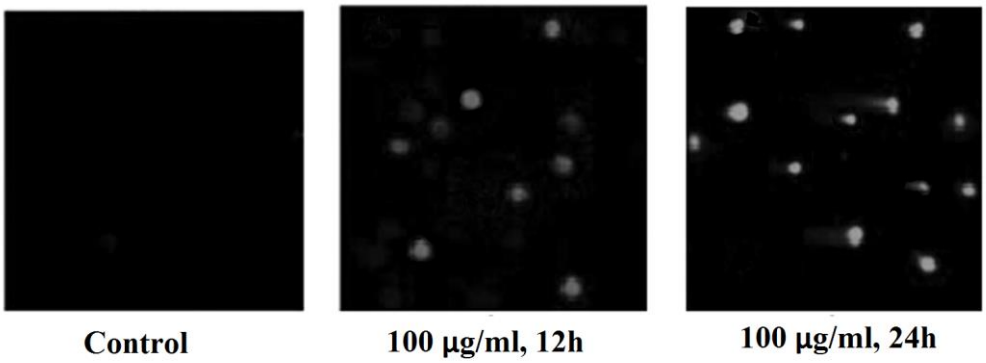

Figure 3. DNA damage assay. A: Immunofluorescence analysis related to phosphorylated H2AX binding to double stranded breaks. Fluorescence intensity demonstrated DNA damage increased in the presence of high concentrations of nanoparticle.

In this study we try to increase our knowledge about the Fe_2O_3-NP and cell interaction by standard methods. According to previous studies the Fe_2O_3-NP induce oxidative stress by ROS overproduction [12, 16, 25]. Recently, Prodan et al. demonstrated that Fe_2O_3-NP was capable of inducing oxidative stress responses in Hella cells [12]. We have studied the toxicity of the Fe_2O_3 nanoparticles on Hep G2 cells. HepG2 cells are hepatoma-derived cell line that preserved many specialized functions of normal liver parenchymal cells (hepatocytes are main cells exposed to the toxicants). This study examined cytotoxic effects of Fe_2O_3-NP on Hep G2 cells by MTT assay and LDH assay was carried out. For this purpose cells were treated with different concentrations (25, 50, 75 and 100 µg/ml) of the suspension of Fe_2O_3-NP in culture medium. Cell viability was determined at 12 h and 24 h after treatment and the test results are shown in figure 1A. These results are in agreement with previous studies that say cell viability decreased by treating with nanoparticle and is concentration and time dependent [12, 13, 16, 19]. We measured LDH leaking from the cells as a different document for cytotoxicity. LDH is an intracellular enzyme that penetrates to the cell culture medium and blood by decreased integrity of cell membrane so it can be used for assessing of the membrane integrity [13, 21]. Figure 1B showed LDH leakage in cells treated by different concentration of nanoparticle. Results revealed enzyme leakage increased in the presence of Fe_2O_3-NP dose and time dependently. By considering high reactivity of nanoparticles, one of the best candidates that damage membrane and increase cell death is oxidative stress [26]. ROS measurement indicates presence of oxidative stress by nanoparticle treatment in a concentration and time dependent manner. Produced ROS can harm the genomic DNA and mitochondria, so can promote the apoptosis process. Our results confirmed DNA double strand breaks in nanoparticle received cells by increased amount of florescence that indicate nanoparticles can reach the nucleus and damage DNA or produced ROS can reach to DNA and break it [25]. Unrepaired DNA damage and other oxidative damages possibly lead cells to the apoptosis [27, 28]. This can be considered as an indirect toxic effect of the nanoparticles. Accumulating evidences suggest nanoparticles can induce cell apoptosis by targeting the mitochondrial apoptosis pathway, which includes activation of loop phosphorylation, cytochrome c release from the mitochondria, decrease in Bcl-2 protein expression, activation of PARP and caspase cascades, and DNA fragmentation that finally cause cell death [27, 29-31].

Comparing with similar studies that were done on the other nanoparticles [22, 26, 31], there isn't similar comprehensive study about the Fe_2O_3-NP toxicity mechanism. Evaluation of studies in this field revealed physical properties such as size of the nanoparticles is more important than nature of it in biological function and toxicity. Our results also suggested application of Fe_2O_3-NP in medicine and detection methods should be limited but by considering its cytotoxicity it could be used in cancer treatment or as bactericidal agents.

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