Ultrasmall Fe$_3$O$_4$ nanoparticles induce S-phase arrest and inhibit cancer cells proliferation

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Abstract: The ultrasmall nanoparticles easily lead to a more seriously response than larger nanoparticles because of their physicochemical features. It is essential to understand their cytotoxicity effects for their further application. Here, we used ultrasmall 9 nm Fe$_3$O$_4$ NPs to explore its cytotoxicity mechanism on breast cancer cells. We demonstrated 9 nm Fe$_3$O$_4$ NPs was effectively internalized into cells and located in nucleus, subsequently, it inhibited DNA synthesis through inducing S-phase arrest. Moreover, 9 nm Fe$_3$O$_4$ NPs induced ROS production and oxidative damage by disturbing the expression of antioxidant-related genes (HMOX-1, GCLC and GCLM), which resulted in the enhancement of cells apoptosis and inhibition of cell proliferation, suggesting its potential to be used as therapeutic drug.

Keywords: breast cancer, nanoparticles, S-phase arrest, reactive oxygen species, gene expression

1 Introduction

The nanoparticles exhibit increased application in biomedical fields such as contrast agent, disease detection and drug delivery [1–3], but their interaction with cell remain poorly understood, especially for the ultrasmall nanoparticles. Owing to cells have a diameter range 10 to 100 µm, cellular parts are much smaller, and proteins are even smaller with a typical range of just 5 to 50 nm [4], therefore the smaller the nanoparticles size, the more the interaction with cellular components, which easily induce more toxic effect than larger particles of the same materials because of larger surface area, enhanced chemical reactivity and easier cellular penetration [5–7].

Fe$_3$O$_4$ NPs lies in its promising application in these fields of tumor diagnosis and drug delivery due to their physicochemical features, including biocompatibility and magnetic properties [8–10]. Therefore, we used ultrasmall Fe$_3$O$_4$ nanoparticles (Fe$_3$O$_4$ NPs) with 9 nm size to investigate the interaction mechanism of ultrasmall nanoparticles with MCF-7 breast cancer cell. We discovered that the Fe$_3$O$_4$ NPs with 9 nm size, it could inhibit the proliferation through enhancing oxidative stress apoptosis of MCF-7 breast cancer cell and disturbing cell cycle (Figure 1). This research contributes to further illustrate the mechanism of Fe$_3$O$_4$ NPs inhibiting MCF-7 breast cancer cells, and suggests the Fe$_3$O$_4$ NPs alone have potential as an antitumor drug to kill tumor.

2 Experimental details

2.1 Materials

Fe (acac)$_3$ was purchased from Acros Organics (Geel, Belgium). Fetal bovine serum (FBS), Dulbecco modified eagle medium (DMEM), Streptomycin, Trizol reagent and trypan blue were purchased from Thermo Fisher Scientific (Waltham, MA, USA). Cell-Light EdU DNA cell proliferation kit was purchased from Guangzhou Ribo-
9-nanometer Fe$_3$O$_4$ NPs were prepared using the polyol method [11]. Briefly, 2 mmol Fe(acac)$_3$ and 25 mL triethylene glycol was directly added into a three-neck round-bottomed flask equipped with condenser, magnetic stirrer, thermograph, heating mantle and stirred under argon. The mixture was heated to 180°C at a rate of 3°C min$^{-1}$ for maintaining 30 min, then was quickly heated to 280°C for another 30 min of reflux. After cooling down to room temperature, a black homogeneous colloidal suspension contained of magnetite nanoparticles was obtained. Then, 2 mmol Fe(acac)$_3$ was again added to react according the aforementioned condition. The obtained solution was dialysis in distilled water and then was collected with a magnetic to obtain 9 nm Fe$_3$O$_4$ NPs. Subsequently, the Fe$_3$O$_4$ NPs was characterized using transmission electron microscope (TEM), dynamic light scattering (DLS) and X-ray diffractometer (XRD).

### 2.3 Cell viability

Cell viability was determined using cell count method [12]. MCF-7 breast cancer cells ($4\times10^4$ cells/well) were seeded in 12-well plate and were cultured for 24 h. Different concentration of Fe$_3$O$_4$ NPs with 9 nm particle size were added and were separately incubated for 24 h, 48 h and 72 h. Cells were then harvested and resuspended in 1 mL PBS and were counted manually with 0.4% Trypan blue in a haemocytometer chamber.

### 2.4 Cell proliferation imaging

Cell proliferation was imaged using EdU cell proliferation imaging kit [13]. Briefly, MCF-7 breast cancer cells ($4\times10^4$ cells/well) were seeded in 96-well plate and were incubated for 24 h. Different concentration of Fe$_3$O$_4$ NPs with 9 nm particle size were added and were incubated for 48 h. The cells were then treated with 50 mM EdU culture medium for 2 h and washed twice (5 min/once) with PBS, followed by incubation with 4% PFA for 30 min. After removing the solution from the wells, 50 uL glycine (2 mg/mL) was added and were incubated for 5 min. Next, 100 uL PBS solution containing 0.5 % Triton X-100 was added and were incubated for 10 min. Thereafter, 100 uL Apollo® staining reaction liquid was added and were incubated for 30 min. Finally, cells were incubated with 100 uL of Hoechst 33342 for 30 min, washed...
three times (5 min/once), and imaged using an IX-51 fluorescence microscope from Olympus Optical Company, Ltd (Tokyo, Japan). Data of cell proliferation was then calculated using Image-Pro Plus 7.0 software from Media Cybernetics, Inc. (Rockville, USA).

2.5 Cell apoptosis

Cell apoptosis was measured using Annexin V/PI apoptosis detection kit. Briefly, MCF-7 cells (1.6×10^5 cells/well) were seeded into 6-well plate and were incubated for 24 h. Different concentration of Fe₃O₄ NPs with 9 nm particle size were added and were incubated for 48 h. Cells were then collected and fixed in a 0.1 M PBS solution containing 4% paraformaldehyde and 5% glutaraldehyde. Cells were then collected and were washed with PBS, trypsinized, harvested, and resuspended in 500 µL stationary liquid containing 2.5% glutaraldehyde and 5% gluteraldehyde. Cells were embedded in agar gel and were then cut into 1-mm slices. Each slice was again fixed using osmic acid, dehydrated, embedded, and imaged using TEM.

2.6 Cell cycle

MCF-7 breast cancer cells (1.6×10^5 cells/well) were seeded into 6-well plate and were incubated for 24 h. Different concentrations of Fe₃O₄ NPs with 9 nm particle size were added and incubation for 48 h. Cells were then collected and fixed in 70% ethanol at 4°C overnight. Cells were then resuspended in PBS and were incubated at 37°C for an additional 30 min at 37°C to allow complete deesterification of intracellular diacetates. Cells were then harvested by trypsinization and at least 2×10⁵ cells from each sample were analyzed using flow cytometry.

2.7 Intracellular distribution

Intracellular distribution of Fe₃O₄ NPs was evaluated using TEM [13]. MCF-7 breast cancer cells were cultured in 10 cm plate and were incubated for 24 h. Fe₃O₄ NPs with 9 nm particle size was added to the plate and were incubated for 3h, cells were then washed with phosphate buffered saline (PBS) and were fixed in a 0.1 M PBS solution containing 2.5% glutaraldehyde for 30min. Afterward, cells were collected and were washed with PBS, trypsinized, harvested, and resuspended in 500 µL stationary liquid containing 4% paraformaldehyde and 5% gluteraldehyde. Cells were embedded in agar gel and were then cut into 1-mm slices. Each slice was again fixed using osmic acid, dehydrated, embedded, and imaged using TEM.

2.8 GSH analysis

Total intracellular glutathione (GSH) levels were measured using Total Glutathione Assay Kit [14]. Briefly, MCF-7 cells (1.6×10^5 cells/well) were seeded into 6-well plate and were incubated for 24 h. Different concentration of Fe₃O₄ NPs with 9 nm particle size were added and were incubated for 48 h. Cells were then washed with PBS, freezed thawing rapidly twice using liquid nitrogen and 37°C water, and were centrifuged at 10,000 × g for 10 min. The supernatant was reacted with DTNB (5,5'-Dithiobis(2-nitrobenzoic acid)) for 5 min at 25°C to obtain the yellow-colored product. Afterwards, the product was assayed at 412 nm absorbance using a microplate reader (Bio-Tek, USA). The GSH concentrations were determined by comparison with standards.

2.9 ROS analysis

Intracellular reactive oxygen species (ROS) levels were assayed using FACSCalibur flow cytometer [14]. Briefly, MCF-7 breast cancer cells (1.6×10^5 cells/well) were seeded into 6-well plate and were incubated for 24 h. Different concentration of Fe₃O₄ NPs with 9 nm particle size were added and were incubated for 48 h. Then, cells were washed with FBS free medium and were incubated with 10 μM DCFH-DA for 30 min at 37°C in the dark. Subsequently, cells were washed twice with FBS free medium to remove the additional dye, and were incubated in FBS free medium for an additional 30 min at 37°C to allow complete deesterification of intracellular diacetates. Cells were then harvested by trypsinization and at least 2×10⁵ cells from each sample were analyzed using flow cytometry.

2.10 qRT-PCR analysis

The mRNA levels of genes involved in oxidative stress (HMOX1, GCLC and GCLM) were measured using quantitative the Applied Biosystems StepOnePlus™Real-Time PCR System (qRT-PCR) (Life Technologies, USA). Briefly, MCF-7 cells (1.6×10^5 cells/well) were seeded into 6-well plate and were incubated for 24 h. Different concentration of Fe₃O₄ NPs with 9 nm particle size were added and incubated for 48 h. Cells were lysed with the addition of trizol reagent. RNA was extracted and purified utilizing standard phenol/chloroform extraction procedures. cDNA was obtained using a mixture containing 5 × PrimeScript RT Master Mix (Takara), Total RNA, and RNase Free dH₂O. Subsequently, qRT-PCR analysis was performed using qRT-
PCR with the SYBR Premix Ex Taq Perfect Real Time Kit. The PCR reaction consisted of initial thermal activation at 95°C for 30 s and 40 cycles. Each cycle was as follows: 95°C for 5 s; 60°C for 34 s. PCR products were verified by analysis of melt curves and amplification plots. Quantitative values were acquired from linear regression of the PCR standard curve. The primer sequences of the amplified genes are as follows: HMOX1 (Heme oxygenase-1), forward 5'-TGGAGACTCCACAGGGAAG-3' and reverse 5'-CACCGGAAAGTTCATGGC-3'; GAPDH, forward 5'-GGATGCAGAAGGAGATCACTG-3' and reverse 5'-CGATCCACACGGAGTACTTG-3', GCLC(glutamate-cysteine ligase, catalytic subunit), forward 5'-TGCACAATAACTTCATTTCCCAGT-3' and reverse 5'-ATCCGGCTTAGAAGCCCTTG-3', GCLM(glutamate-cysteine ligase, modifier subunit), forward 5'-GGTCAGGGAGTTTCCAGATGT-3' and reverse 5'-CTGTGCAACTCCAAGGACTGA-3'.

2.12 Statistics
Data were presented as mean ± standard deviation (S.D.) of at least three independent experiments. The significance of differences in data of different groups were appropriately determined by the unpaired Student’s t-test at $P<0.05$.

3 Results and discussion

3.1 Characterization of Fe$_3$O$_4$ NPs
In order to research the relative mechanism of toxicity of ultrasmall Fe$_3$O$_4$ NPs in MCF-7 cells, we synthesis the Fe$_3$O$_4$ NPs using the polyol method. XRD result suggest that the observed diffraction pattern can be indexed to Fe$_3$O$_4$ (JCPDS file 19-0629), demonstrating the Fe$_3$O$_4$ nanoparticles are successfully synthesized. TEM and DLS results verify that Fe$_3$O$_4$ NPs exhibit a spherical morphology and a diameter of 9 nm, and they have uniform and good size distribution (Figure 2A, 2B).
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3.2 Cell viability and cell proliferation imaging

Cell count method was employed to evaluate the effect of 9 nm Fe$_3$O$_4$ NPs for cell growth viability. As shown in Figure 3A, cell growth number gradually increased with the extension of incubation time from 24h to 72h in the control group alone. As the increase of Fe$_3$O$_4$ NPs incubation concentration from 10 µg/mL to 1000 µg/mL, cell growth number exhibited a concentration-dependent decrease. Especially at 1000 µg/mL incubation concentration, it reduced the cells number to the minimum, demonstrating that the Fe$_3$O$_4$ NPs affect the cells number in a concentration-dependent manner.

Cell proliferation depends on both cell division and cell death [15]. EdU cell proliferation imaging was carried out to further illustrate the influence mechanism of Fe$_3$O$_4$ NPs for cell proliferation. EdU easily inserted to DNA in the course of DNA synthesis, it thereby is employed to label S-phase cells for monitoring DNA synthesis and trace of the EdU-labelled cells when they progressed through the cell cycle and divided. As indicated in overlap image of Figure 3B, a gradually diminishing red fluorescence in quantify were observed in MCF-7 cells exposed to an increasing Fe$_3$O$_4$ NPs concentration from 10 µg/mL to 1000 µg/mL, demonstrating that Fe$_3$O$_4$ NPs disturb DNA synthesis of MCF-7 cells in a concentration-dependent manner and thereby further decrease cell proliferation (Figure 3C), which is consistent with the result of cell growth number. Especially for the high concentration of Fe$_3$O$_4$ NPs, it exhibited the most obvious interference efficiency. These experimental results suggested that the difference in concentration of nanoparticles induces different toxicity response.

3.3 Cell apoptosis

Cell apoptosis assay was performed to illustrate the mechanisms of Fe$_3$O$_4$ NPs affecting MCF-7 cells growth and proliferation, in which two different concentrations of Fe$_3$O$_4$ NPs was employed. As shown in Figure 4A and 4B, compared with control group, 15 µg/mL Fe$_3$O$_4$ NPs-treated cells exhibited no less low survival rates, and the early and
Figure 4: The analysis of cell apoptosis and cell cycle: Flow cytometer analysis on cell apoptosis of MCF-7 cells incubated with 9 nm Fe$_3$O$_4$ NPs for 48 h at a concentration of 15 µg/mL (A) and 600 µg/mL (B). Flow cytometer analysis on cell cycle of MCF-7 cells with 9 nm Fe$_3$O$_4$ NPs for 48 h at a concentration of 15 µg/mL (C) and 600 µg/mL (D). All histograms are quantitative figures for the corresponding cycle and apoptosis. Data are presented as mean ± S.D. (n=3)
late apoptosis of cells treated with 15 µg/mL Fe\textsubscript{3}O\textsubscript{4} NPs also were no obviously variation, suggesting a negligible influence of Fe\textsubscript{3}O\textsubscript{4} NPs for cell apoptosis at the incubation concentration of 15 µg/mL.

However, as the concentration increasing to 600 µg/mL, the survival rate of cells treated with Fe\textsubscript{3}O\textsubscript{4} NPs obviously decreased as compared to the control. The early apoptosis of cells treated with Fe\textsubscript{3}O\textsubscript{4} NPs approximated 30%, which were obviously upregulated as compared to the control. For the late apoptosis, Fe\textsubscript{3}O\textsubscript{4} NPs-treated cells was upregulated significantly to 60% as compared to the control, indicating that concentration of Fe\textsubscript{3}O\textsubscript{4} NPs obviously affect cell apoptosis, which predominantly result from disturbing the early and late apoptosis of cells.

3.4 Cell cycle

Cell cycle involves DNA replication and cell separation, consisting of four distinct phases: G1 phase, S phase, G2 phase and M phase. The cell cycle deregulation can induce cell apoptosis [16–18]. We carried out this experiment to illustrate the mechanism of Fe\textsubscript{3}O\textsubscript{4} NPs interfering cell cycle. As can be seen from Figure 4C and 4D, G2/M, S and G0/G1 of cells exposed to Fe\textsubscript{3}O\textsubscript{4} NPs with the concentration of µg/mL were regular and were no obviously change as compared to the control, cell cycle phase can be ranked in increasing order: G2/M>S>G0/G1. As the concentration increasing to 600 µg/mL, the cycle of cells exposed to Fe\textsubscript{3}O\textsubscript{4} NPs was disrupted, about 60% and 20% of cell population were separately at S and G0/G1 phase, a primarily S-phase cell-cycle arrest was confirmed. For further exploring the mechanism of S-phase cell-cycle arrest, we performed the intracellular distribution experiment of Fe\textsubscript{3}O\textsubscript{4} NPs. As shown in Figure 5A, Fe\textsubscript{3}O\textsubscript{4} NPs with the concentration of 600 µg/mL internalized by cells mostly distributed in autophagosome and nuclear of cells. Whereas the nanoparticles in the cell nucleus could affect the DNA synthesis, and potentially induced apoptosis, thereby decreased cell proliferation accordingly.

Figure 5: The production of GSH and ROS induced by nanoparticles: (A) Intracellular distribution image of Fe\textsubscript{3}O\textsubscript{4} NPs in cells exposed to Fe\textsubscript{3}O\textsubscript{4} NPs (600 µg/mL) for 3 h. The corresponding image (right) is the magnified image of regional image in A figure. (B) GSH level of cells treated with Fe\textsubscript{3}O\textsubscript{4} NPs for 48h. The ROS images of cells treated separately with control (C) and 9 nm Fe\textsubscript{3}O\textsubscript{4} NPs (D) for 48h. Data are presented as mean ± S.D. (n=3)
3.5 Intracellular glutathione and ROS production

Glutathione (GSH) represents the major intracellular redox buffer in cells, it is the first line of cellular defense mechanism against oxidative injury when nanoparticles was internalized by cells [19–21]. The experimental results were shown in Figure 5B, Fe$_3$O$_4$ NPs induced a dose-dependent decrease of GSH concentration in MCF7 cells as compared to the control alone. Especially at the concentration of 1000 µg/mL, the GSH level of cells treated with Fe$_3$O$_4$ NPs significantly decreased to about 5 µM, which induced a reduced by 92% compared to control.

Owing to ROS generation could directly cause oxidative injury and play an important role in cell signaling and regulate inflammatory responses, toxicity of drugs, apoptosis or programmed cell death [22, 23], we then assayed the expressed level of reactive oxygen species (ROS). As can be seen from Figure 5C and 5D, compared with the control, a high green fluorescence intensity was observed in cells treated with Fe$_3$O$_4$ NPs with the concentration of 1000 µg/mL, demonstrating that Fe$_3$O$_4$ NPs induce higher ROS production in MCF-7 cells. In combination with the experimental result of GSH, it can be concluded that the low GSH level induce high ROS expression, and thus result in the oxidative damage and cause cell apoptosis.

3.6 Expression of anti-oxidative genes and proteins

To further investigate the mechanism of Fe$_3$O$_4$ NPs inducing oxidative response in MCF-7 cells, the mRNA expressions of three antioxidant-related genes (HMOX-1, GCLC and GCLM) and their protein levels were measured [24]. We found that the expressions of HMOX-1 mRNA, GCLC mRNA, and GCLM mRNA in cells with treated Fe$_3$O$_4$ NPs were upregulated when compared with the control, moreover, a gradually increasing trend was observed with the incubation concentration enhancing from 10 to 500 µg/mL (Figure 6A). When the incubated concentration ranges from 500 µg/mL to 1000 µg/mL, the expression of HMOX-1 mRNA, GCLC mRNA, and GCLM mRNA in cells treated with Fe$_3$O$_4$ NPs declined, suggesting that the difference of the concentration induce the different toxicity response.

The expressions of HMOX-1, GCLC, and GCLM protein were then determined using western blotting (Figure 6B). The HMOX1 protein expression level in cells treated with Fe$_3$O$_4$ NPs was weaker than the GCLC and GCLM protein expression level at the incubation concentration from 0 to 100µg/mL, suggesting that the Fe$_3$O$_4$ NPs induce an oxidative damage that is mainly mediated by disturbing the protein expressions of GCLC and GCLM. However, we also noticed that the expressions of HMOX-1, GCLC and GCLM protein exhibited a minimum expression at the incubated concentration of 1000 µg/mL, which was attributed to a low protein synthesis caused by a high oxidative damage and contributed to induce an effective inhibition for MCF-7 cell growth.

4 Conclusions

In summary, we demonstrated the ultrasmall 9nm Fe$_3$O$_4$ NPs effectively inhibited DNA synthesis and enhanced cell
apoptosis by inducing S-phase arrest, which thereby reduced the cell growth and proliferation. In addition, 9nm Fe₃O₄ NPs remarkably disturbed the expressions of HMOX-1 mRNA, GCLC mRNA, and GCLM mRNA, inducing the high ROS production and decreased GSH, leading to a seriously oxidative damage for MCF-7 cells, which make 9 nm Fe₃O₄ NPs inhibit the growth of MCF-7 cells. These results suggesting 9 nm Fe₃O₄ NPs have potential to be used as the antitumor drug.

Conflict of Interests: The authors declare no conflict of interest regarding the publishing of this paper.

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