

Optimal Concentration of PEG-Coated Fe₃O₄ Nanoparticles for Generation of Reactive Oxygen Species in Human-Derived Amniotic Membrane Stem Cells

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Abstract

Introduction: Superparamagnetic iron oxide nanoparticles (SPIONs) are widely used for various biomedical applications. Although the potential benefits of SPIONs are considerable, it has been shown that SPION uptake can cause intracellular stress via the generation of reactive oxygen species (ROS). Therefore, it is necessary to find the proper dose of SPIONs for bio medical applications and treatment and diagnosis. The main goal of this study was to find a concentration of SPIONs that can induce low intracellular levels of ROS. **Methods:** After synthesis SPIONs were incubated with human-derived amniotic membrane stem cells (hAMCs) in 5-200 µg/mL concentrations. Level of ROS was

then measured using fluorimetry with Rhodamine 123 oxidation-sensitive fluorescent probe uploading. **Results:** Data analysis showed that there was no significant increase in generation of ROS in the cells with the particle concentration range of 5-100 $\mu\text{g}/\text{mL}$. However, at concentrations higher than 100 $\mu\text{g}/\text{mL}$, generation of ROS increased. **Conclusion:** SPIONs coated with polyethylene glycol (PEG) in concentrations ≤ 100 $\mu\text{g}/\text{mL}$ have excellent labeling efficiency and biocompatibility for hAMCs. Higher SPION concentrations induced the generation of ROS in labeled cells.

Keywords: hAMCs, cell labeling, iron oxide nanoparticles, ROS

Introduction

Superparamagnetic iron oxide nanoparticles have been approved for medical applications, diagnosis and cell therapy [1]. Factors such as size, shape and surface charge of nanoparticles can determine their cellular internalization and distribution as well as their efficacy. The requirements for use of nanoparticles in biomedical applications include high magnetization value, size ≤ 100 nm, narrow particle size distribution and specific surface coating [2]. Various materials have been evaluated for SPION surface coating such as polymers, biomolecules, metals and silica to make them more biocompatible. PEG coating improves labeling efficiency with subtle distribution of SPION complexes in cells [1]. The biological toxicity of nanoparticles highly depends on the organ or cell types exposed primarily. The toxicokinetics of uptake, transport, metabolism and accumulation of nanoparticles in different organs and their final excretion are equally important when discussing nanotoxicity [3, 4]. Iron overload due to the degradation of SPIONs has also been presumed to cause cytotoxicity. Several adverse effects such as generation of ROS, membrane and DNA damage have been reported in SPION-labeled cells [1, 5]. ROS, as the name implies, derive and present higher reactivity than molecular oxygen with redox activity [6, 7]. The recent interest in the role of free radical processes in cellular physiology and pathology has resulted in a demand for methods of quantification of ROS production in living cells. Thus, detection of ROS relies on detecting end products formed when specific compounds react with ROS. These include measurements of oxidation of dichlorofluorescein (H2DCF), Rhodamine 123 (RH123) or hydroethidine (DHE). Rhodamine 123, an anionic dye, is capable of penetrating into the mitochondrial matrix and causing photoluminescence quenching dependent on mitochondrial transmembrane potential [8]. In fact, RH123 can be oxidized by various ROS thereby serving as an indicator of the degree of general oxidative stress [9]. This study aimed to evaluate the effect of nanoparticles on hAMCs because these cells have anti-inflammatory properties, secrete growth factors, have high differentiation rate and are easily accessible and affordable [10]. Thus, further studies are required to assess the effect of iron oxide nanoparticles on these cells to find a non-toxic dose of these nanoparticles for use to label and track cells with MRI after injection into the target tissue. This study aimed to evaluate

the effect of different concentrations of PEG-coated SPIONs on hAMCs. Because we want to monitor the in vivo migration of labeled hAMCs with MRI after intra-myocardial injection in the next study.

Materials and Methods

Particle preparation:

Nanoparticles were synthesized by classic co-precipitation of iron oxide in an alkaline solution in the presence of polyethylene glycol. Next, magnetic nanoparticles were characterized by various physicochemical means such as transmission electron microscopy (TEM), dynamic light scattering (DLS) and infrared spectroscopy (FT-IR).

Isolation and cultivation of human mesenchymal cells from the amniotic sac:

Term placentas from pregnancies with normal evolution were taken via normal delivery without using any invasive method (Milad Hospital) after obtaining written informed consent of the pregnant women. The harvested placentas were transported in cold phosphate buffered saline (PBS) solution in a thermally insulated container on ice. To obtain mesenchymal cells, the amniotic membrane was mechanically separated from the subjacent chorion by detachment. The membrane fragments were transferred to Petri dishes and blood vessels were removed. The tissue was washed with PBS solution and mechanically crushed by a blade to obtain quite small pieces of tissue. The pieces of tissue were digested by collagenase and incubated for four hours. Then, they were centrifuged at 1500×g for five minutes and the pellets were suspended in low glucose Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 2 mM L-glutamine, 100 U/mL penicillin, and 100 µg/mL streptomycin (Gibco Co., Germany). The cells were plated in 25 cm² flasks supplemented with DMEM and maintained in 5% CO₂ atmosphere at 37°C. The medium was refreshed 48-72 hours after the initial culture to remove non-adherent cells and the adherent cells were grown to confluence before each passage. The medium was refreshed twice a week.

Cell characterization by flow cytometry:

In order to ensure that the isolated cell was a mesenchymal cell, we performed flow cytometric assay. Cells were stained with specific antibodies for flow cytometry. In brief, cultured hAMCs were washed twice in PBS and harvested using 0.25% trypsin/EDTA. The cells were then washed with PBS and divided into groups for antibody staining. Each aliquot contained approximately 5 × 10³ cells. The antibodies were used to detect cell surface antigens namely CD90, CD105, CD166, CD45 and CD34. All antibodies were conjugated with fluorescein-isothiocyanate and phycoerythrin. The cells were stained at 4°C for 30 minutes. After incubation, the cells were washed with PBS and re-suspended in 500 µL of PBS. Analysis was performed with a FACSCalibur flow cytometer (BectonDekenson, USA).

Osteogenic Differentiation:

Osteogenic differentiation was performed with medium DMEM-HG supplemented with 10 μ M dexamethasone, 10 nM vitamin D3, and 50 mg ascorbic phosphate for 21 days. Calcium deposits were evidenced by 2% Alizarin Red staining for 10 minutes PBS solution. Then viewed with invert microscop.

Adipogenic differentiation:

Cells were cultivated for 21 days in DMEM-HG supplemented with 1 μ M dexamethasone, 10 μ g/mL insulin, and 200 μ M indomethacin. The cells were stained with 0.5% Oil Red O for 10 minutes to reveal the intracellular accumulation of lipid-rich vacuoles. Then viewed with invert microscop.

All reagents used in this experiment were from Sigma Co.

Cell culture:

Human derived amniotic membrane stem cells were cultured in DMEM supplemented with 10% FBS at 37°C and 5% CO₂ in a humidified atmosphere. Cells were subcultured upon reaching 80% to 90% confluence. All experiments were performed on third-fifth passage cells.

Magnetic cell labeling:

SPIONs were added directly to the cell culture at concentrations of 0,5, 10, 20, 30, 40, 50, 75, 100, 125, 150, 175 and 200 μ g /mL. After 48 hours of incubation with cells, culture media were removed and cells were washed three times with PBS buffer to wash out non-internalized particles and use them for subsequent assays.

ROS assay:

Treated cells were collected and resuspended in PBS, which contained 10 μ M Sigma RH123 and incubated at 37 °C for 30 minutes. When the mitochondrial membrane potential depletes, the RH123 is released from the mitochondria and produces fluorescence. Samples were analyzed by fluorimetry; which was performed with an excitation wavelength of 422 nm and an emission wavelength of 520 nm.

Statistical analyses:

All values were expressed as mean \pm standard deviation from three experiments. Statistical analyses were performed using SPSS version 21.0. Data were analyzed for statistical significance by one-way ANOVA with post hoc Bonferroni correction for multiple comparisons. A P-value of less than 0.05 was considered statistically significant.

Results

Particle preparation:

PEG-coated SPION samples were successfully obtained and had spherical morphology and average particle size of 20 nm. Morphological examinations of the nanoparticles were performed using a TEM ([Figure 1](#)) and the diameter of the nanoparticles was analyzed by DLS ([Figure 2](#)). Also, FT-IR analysis of SPIONs and surface-modified SPIONs was performed to characterize the surface nature of these particles and study the coating properties of the magnetite surface ([Figure 3](#)).

Cell harvesting from the amniotic membrane:

We succeeded in isolating and culturing mesenchymal cells from the placental amniotic membrane. At first, the cells had a round shape and as this process progressed they became elongated, finally acquiring a typical fibroblastic morphology ([Figure 4](#)).

Cell characterization by flow cytometry:

We conducted flow cytometric analysis of the third passage cells and monitored the expression of five CD markers (CD34, CD45, CD90, CD105 and CD166) to determine whether the cells displayed a mesenchymal stem cell phenotype. The cells that expressed CD90, CD166 and CD105 showed a negative reaction to hematopoietic markers such as CD34 and CD45 ([Figure 5](#)).

Osteogenic Differentiation and Adipogenic differentiation:

Adipogenic differentiation of hAMCs was apparent after 21 days of incubation with adipogenic medium. By the end of the third week, half of the cells contained Oil red O-positive lipid droplets. The colonies of hAMCs were subjected to Alizarin red staining 21 days after the initiation of osteogenic differentiation. Intense Alizarin red staining of the colonies confirmed that calcium deposition had occurred ([Figure 6](#)).

ROS assay:

Different concentrations of SPIONs had cytotoxic effects on hAMCs compared to the control group after 30 minutes of incubation. The generated ROS was 0.58%±0.53%, 0.73%±0.24%, 0.88%±0.23%, 1.48%±1.26% , 2.15±0.77, 3.32 ± 0.72, 6.96± 1.19, 13.17± 0.83 (P<0.05), 25.63± 0.91 (p<0.005), 34.58± 5.44 (p<0.005), 49.96± 1.49 (p<0.005) and 56.05± 1.54 (p<0.005) at iron concentrations of 5, 10, 20, 30, 40, 50, 75, 100, 125, 150, 175 and 200 µg /mL, respectively. The suitable dose of SPION for use on hAMCs was calculated to be 100 µg /mL ([Figure 7](#)).

Discussion

Superparamagnetic iron oxide nanoparticles are important for medical applications. Despite extensive applications of these nanoparticles, exposure of cells to these nanoparticles may induce the release of ROS in cells [11, 12]. The toxicity of SPIONs is strongly dose-dependent. Moreover, it depends on the way it is expressed i.e. mass versus particles versus surface area. The choice of more biocompatible iron oxide-based magnetic particles would resolve the problem of cytotoxicity. Coating of these nanoparticles with an additional biocompatible layer may improve the colloidal stability and biocompatibility [13]. We used PEG for coating of SPIONs. At low levels of ROS production, cells initiate a protective response to guarantee their survival, but excessive production of ROS can initiate cellular tissue damage by modifying and denaturing lipids, proteins and DNA, which seriously compromises cell viability and may induce a variety of cellular responses via the generation of secondary reactive species, leading to cell death by necrosis or apoptosis [11, 12]. We demonstrated that intracellular oxidative stress indicated by ROS production in SPION labeled cells occurred in a concentration range from 5 to 200 $\mu\text{g}/\text{mL}$, which was detected using Rhodamine 123. Our data indicated that SPION labeling dose-dependently affected hAMCs in vitro and we showed that the PEG coating of SPION was not toxic to cells at concentrations less than 125 $\mu\text{g}/\text{mL}$ with respect to controls. Furthermore, nanoparticles at concentrations between 5-100 $\mu\text{g}/\text{mL}$ were biocompatible and suitable for use in vivo and for therapeutic purposes. This result was in accord with the findings of a previous experiment on generation of ROS in cells loaded with SPION in vitro. Naqvi et al, in 2010 evaluated the cytotoxicity of iron oxide nanoparticles based on their concentration via assessment of increase in oxidative stress markers. They cultured human macrophages and added nanoparticles to this medium. Using an ascending concentration of iron oxide nanoparticles increased ROS, which subsequently caused cell injury and cell death [14]. Hoskins et al. showed that iron nanoparticles increased the generation of ROS.

Production of oxidative stress factors caused cell death [15]. Studies have shown that iron metabolism damages the SPION surface, increases the concentration of iron ions and induces oxidative stress [16]. Thus, it seems that high dose injection of these nanoparticles causes DNA damage, cell necrosis and apoptosis via oxidative stress [4, 5]. The results of the previous studies as well as the current study showed that cell damage due to iron oxide nanoparticles was dose-dependent. Thus, use of proper dose of iron oxide nanoparticles is very important to prevent cell damage or cell death due to oxidative stress. Our results revealed that generation of ROS in cells labeled with a specific dose of SPION was not significantly different from that in controls. Thus, SPIONs at a specific concentration can be used for detection of cells.

Conclusion

Considering the extensive applications of nanoparticles in medicine, the effects of SPIONs on cells must be evaluated. In this study, we assessed the effects of SPIONs on generation of ROS by measuring the level of ROS in cells treated with various doses of SPIONs. We observed that the intracellular level of ROS gradually increased with an increase in concentration of nanoparticles. Our data may be useful for efficiently calibrating the best SPION dose to minimize their harmful effects on cells.

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Figures:

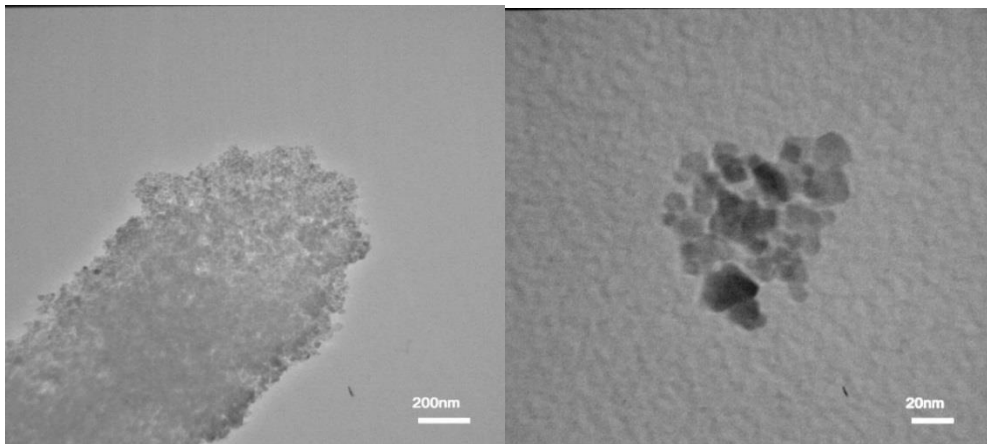


Figure 1. Spherical shape of a synthesized iron oxide nanoparticle under an electron microscope

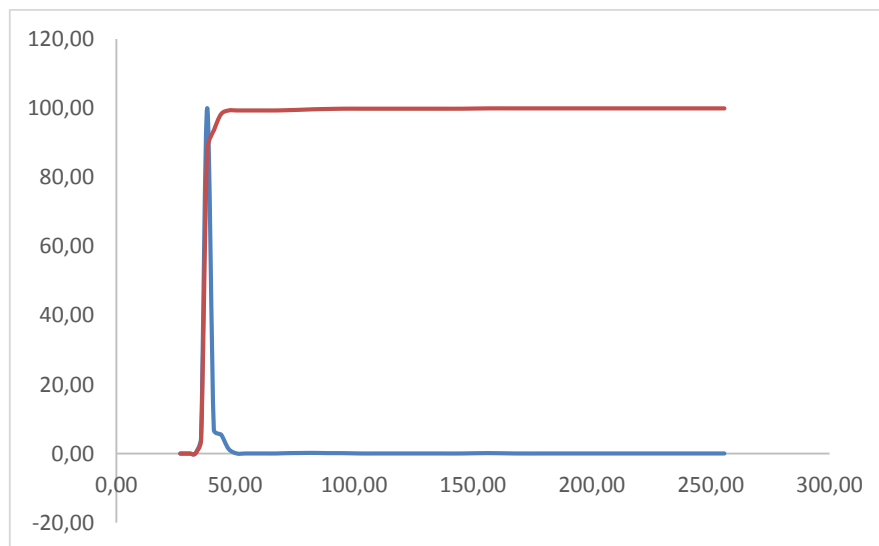


Figure 2. Hydrodynamic size of magnetic nanoparticles

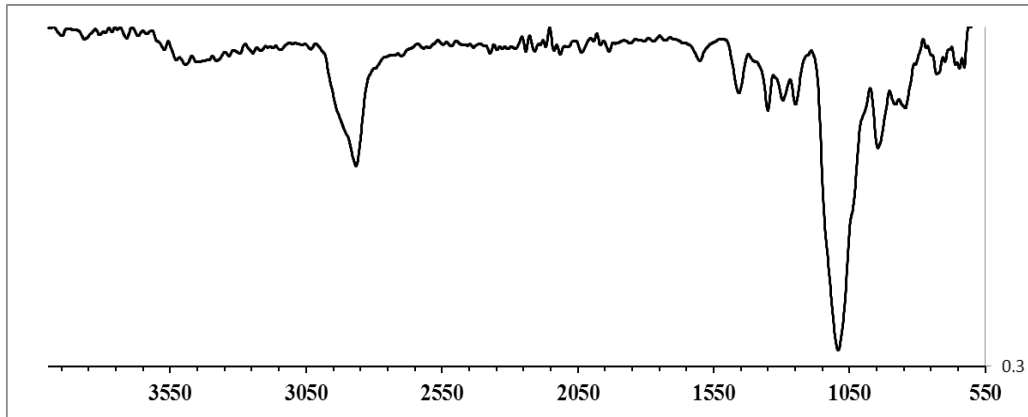


Figure 3. FTIR spectroscopy of spion nanoparticles

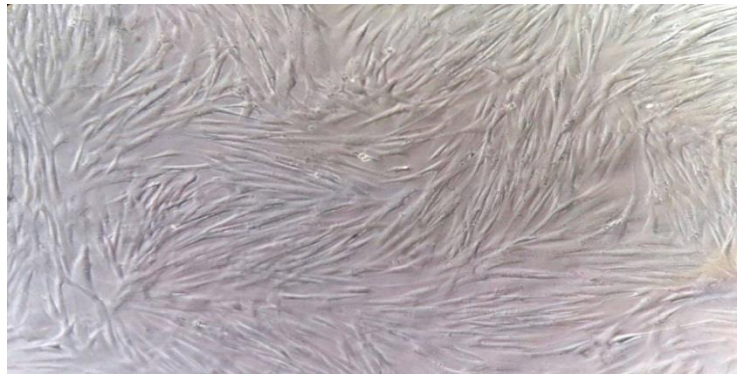


Figure 4. Third passage stem cells isolated from the amniotic membrane at $\times 200$ magnification

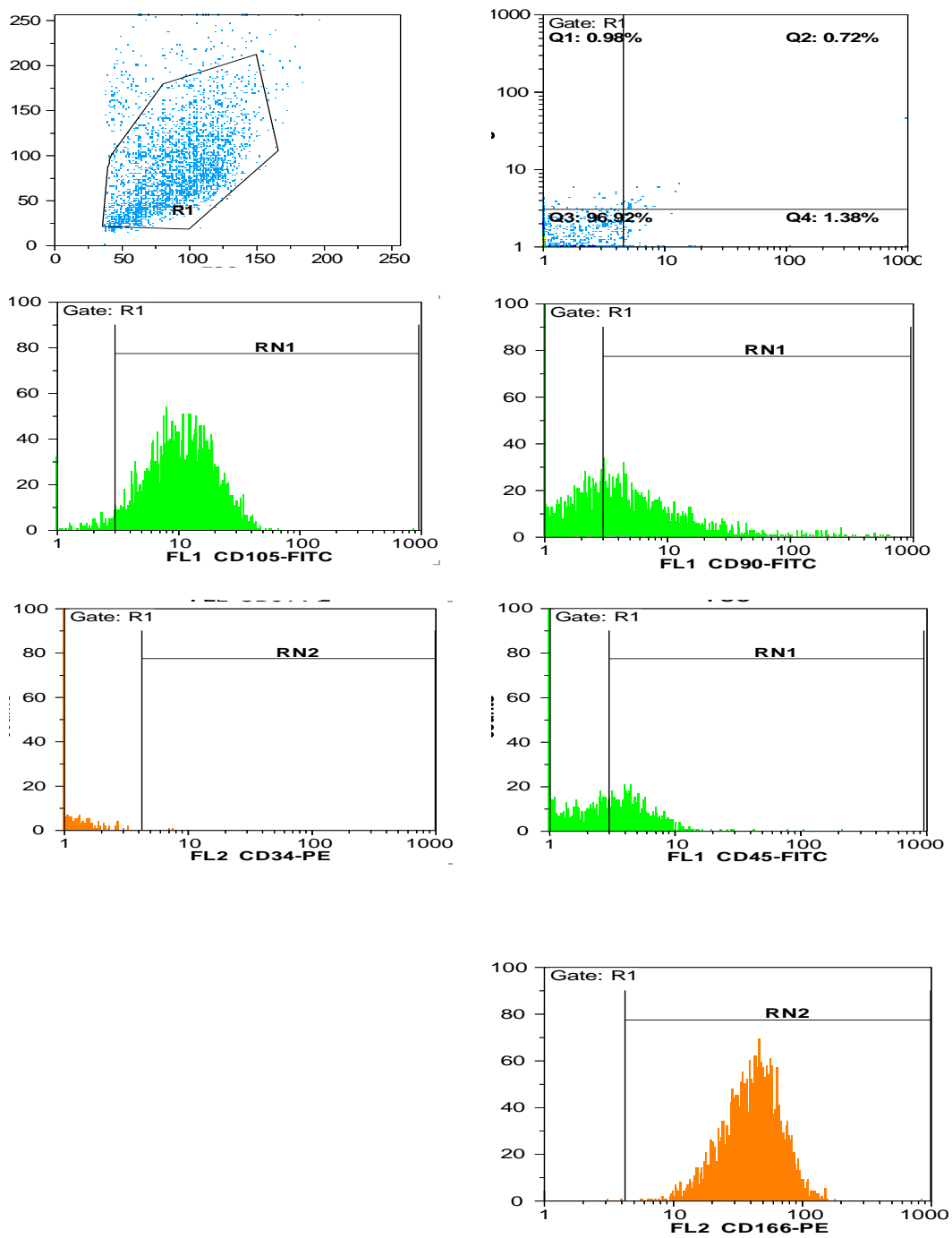


Figure 5. Flow cytometric analysis of hAMCs showing positive surface markers for mesenchymal stem cells and negative surface markers for hematopoietic cells

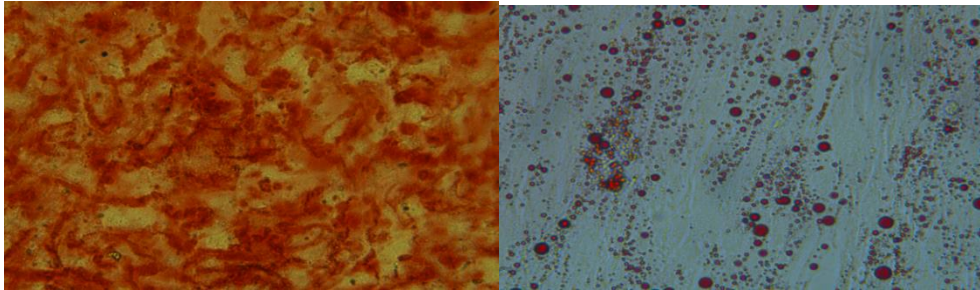


Figure 6. Differentiation capacity of mesenchymal stem cell. Osteogenic differentiation of hAMCs (left) and Adipogenic differentiation of hAMCs (right) was determined after culturing for 21 days under osteogenic and adipogenic conditions.

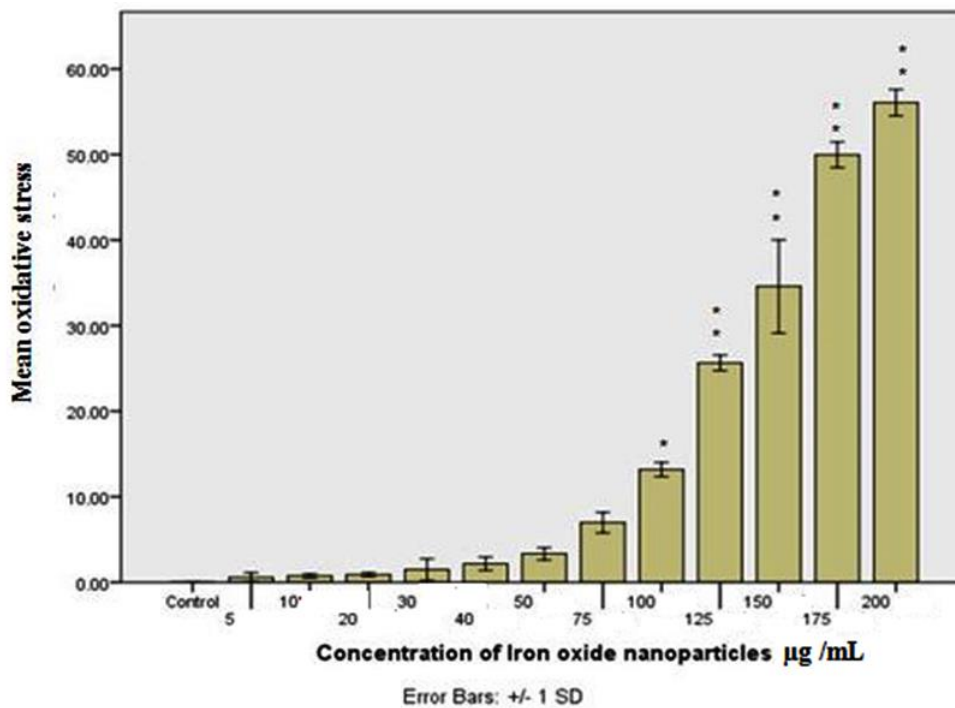


Figure 7. Cytotoxic analysis of SPIONs. Oxidative stress in cells after exposure to various concentrations of SPIONs (5, 10, 20, 30, 40, 50, 75, 100, 125, 150, 175 and 200 µg/mL) for 30 minutes. Data are expressed as mean \pm standard deviation from three experiments as % of control cells. Statistical significance compared to unlabeled cells as controls. *P < 0.05 and **P < 0.005.

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