Original Article

Iron Oxide Nanoparticles Reduced Retinoic Acid Induced-neuronal Differentiation of Mouse Embryonic Stem Cells By ROS Generation

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Abstract

Background: In recent years the increasing use of nanoparticles has led researchers to study their effects on biological systems. The most important effects of nanoparticles on cells are their ability to induce or suppress production of reactive oxygen species (ROS). Changes in reactive oxygen species play an important role in various developmental processes, including proliferation and differentiation in several diseases such as Parkinson. The aim of this study was to investigate the effect of iron oxide nanoparticle with dimensions of less than 20 nanometers on the viability and neuronal differentiation of mouse embryonic stem cell (Royan B1).

Methods: To assess the effects of Fe_2O_3 nanoparticles on neuronal differentiation of Royan B1 cells, embryoid bodies were divided into eight groups receiving different amounts of nanoparticle (10, 20, 30 µg/mL) for 12 hours, retinoic acid (1 µM), and both. Differentiation was examined under phase contrast microscope and using immunocytochemistry.

Results: Data analysis showed that cell death was increased by a time and concentration manner and there was a direct relevance between iron oxide amount and H_2O_2 level in cells. Statistical analysis of embryoid bodies showed that neural differentiation of mouse embryonic stem cells in groups that received nanoparticles were significantly lower than other groups and their viability were considerably reduced.

Conclusion: According to the findings of this study it can be concluded that iron oxide nanoparticles reduce retinoic acid-neuronal differentiation in mouse embryonic stem cells and it seems that the main mechanism involved in the reduction of viability and neural differentiation was enhanced levels of ROS within the cells.

Keywords: Cell toxicity, iron oxide nanoparticles, mouse embryonic stem cell, reactive oxygen species, retinoic acid

Introduction

Nanotechnology can be defined as using nanoscale materials. Due to their small size, nanoscale materials have unique properties.[1] Nanoparticles have been used in biological and medical applications such as clinical diagnostics, therapeutics areas and drug delivery.[2] Power and abilities of nanoparticles encourage scientists to use them in tissue engineering.[3,4] Despite the increasing use of nanoparticles in the engineering, medicine and biology, their risks are still very unclear and debatable. The majority of concerns arise from their small size and high reactivity.[5,6] Nanoparticles can serve as a vehicle to deliver drugs to a target organ and/or tissue across the blood-brain barrier (BBB).[7,8] Many studies have been conducted to evaluate the potential applications of iron oxide nanoparticles in the regeneration of neuronal functions; however, little is known about the influence of iron oxide nanoparticles on the subcellular or molecular level inside cells.[9] Iron oxide nanoparticles can release free iron ions within the acidic lysosomal environment. These ions can be transported into the cell and surrounding milieu. Due to their radiation ability, many nanoparticles such as iron oxide can be used to detect diseases especially tumors. It has been shown that metal ions such as Mn, Co, Al, and Fe can modulate cell attachment and affect neuronal differentiation.[10,11] In this study, the effects of iron oxide nanoparticles on the neuronal differentiation of mouse embryonic stem cells (mESCs; Royan B1 cell line) were investigated. The recent findings that iron oxide nanoparticles possess intrinsic peroxidase-like activity[12] and the important role of H_2O_2 in cell growth made it necessary to investigate whether iron oxide nanoparticles can affect differentiation by changing intracellular H_2O_2.

Materials and Methods

Preparation of nanoparticles

Iron oxide nanoparticles with dimensions of less than 20 nanometers were prepared and encapsulated by a PEG-phospholipid shell to increase their biocompatibility and water dispersibility by Zist Shimy Azma Company (Tehran, Iran). FTIR (Termoseintific, Nicolet iS10, USA) was used to detect changes in chemical bonds in the compounds. The FTIR method is based on measuring the vibration of the molecular bonds in a combination that is stimulated by an appropriate frequency of infrared radiation. The iron oxide nanoparticles were suspended in Kno DMEM cell culture medium (Gibco, USA) and dispersed by an ultrasonic bath (Elima, Germany) for 10 minutes.

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Cell culture and nanoparticle suspension preparation

Mouse embryonic stem cells (Royan B1) were obtained from Royan Institute (Tehran, Iran). The cells were cultured in Kno DMEM supplemented with 15% FBS (Sigma, USA), on the mouse embryonic fibroblast (MEF). The suspension of iron oxide nanoparticles was prepared using the culture media and dispersed for 10 minutes by an ultrasonicator bath to prevent aggregation. Different amounts of the iron oxide nanoparticles (10, 20, 30, 40, 50, and 60 µg/mL) were prepared in cell growth medium.

Cell viability assay

Cell viability was assessed by the MTT assay, which was based on the reduction of the dye MTT to formazan crystals, an insoluble intracellular blue product, by cellular dehydrogenases. The cells were cultured in the medium containing different amounts of the iron oxide nanoparticles (10, 20, 30, 40, 50, 60 µg/mL) for 12 and 24 hours. A culture medium without the iron oxide nanoparticles served as the control in each experiment. This mixture was measured in an ELISA reader (Metertech, Taiwan) with a wavelength of 570 nm. Cell viability was expressed as a percentage of the viability of the control culture.

Determination of intracellular H2O2

To measure the intracellular H_2O_2 level, mESCs after 12 hours treatment with 30, 20, 10, 0 µg/mL iron oxide nanoparticles were incubated for 1hour with 10 mM 2,7-dichlorodihydrofluorescein diacetate (Molecular Probes) at 37ºC in the dark. Then the cells were washed twice and resuspended in PBS; the fluorescence intensity was detected by FACSCalibur flow cytometry and Cell Quest Pro software (Partec PAS, Germany). The mean fluorescence intensity of Ferucarbotran-labeled cells was normalized to that of unlabeled cells as control.

Neuronal differentiation of mESC was induced by RA treatment (1 µM) (Sigma, USA). Cells were plated at a density of 1×10^4 cells/mL and then hanging drops manner was used to form aggregates, termed embryoid body (EB) with 15% FBS. After 2 days, EBs were transferred to non-adhesive bacterial dishes with 10% FBS. EB suspensions were divided into 8 groups (Table 1). Except group 1 and 8 all other groups received iron oxide nanoparticles for 12 hours. After 4 days EBs replated into gelatin-coated 24 wells with 5% FBS for 5 days to analyses neuronal differentiation.

Immunocytochemical staining

Differentiated neural Cells at day 2+4+5 were washed twice with PBS and fixed with 4% Paraformaldehyde (Sigma, USA) for 24 hours at 4ºC. The cells were permeabilized, and blocked in PBS containing 0.2% Triton X-100 (Sigma, USA) and 10% goat serum for 10 min and 30 min, respectively. Then, the cells were incubated in primary antibody (anti-β-tubulin III, 1:250; sigma, T5293), and diluted in 0.5% bovine serum albumin (BSA) (Sigma, USA) at 37ºC for 1 hour. At the end of the incubation period, the cells were washed 2X with PBS+0.05% tween 20 and incubated with the fluorescence isothiocyanate (FITC)-conjugated anti-mouse IgG (1:250; sigma, F9006) diluted with 0.5% BSA for 60 min at 37ºC. After washing twice with PBS+0.05% tween 20, specimens were examined under a fluorescence microscope (BX51, Olympus, Japan). The length and thickness of neuronal processes were measured by Olysia Bioreport software (Olympus, version: 5.1.2600.2180).

Statistical and morphological analysis

For statistical analysis, nearly 100 EBs were observed in each experiment and three or more replicates were performed. All values were expressed as mean ± SE. Percentage of differentiated EBs into neural cells at day 2+4+5, were analyzed by the Mann-Whitney test, while student t-test (SPSS software) was used for measuring the length and thickness of neural processes. The significant differences between the treatments (*P*-value) were defined as $P < 0.05$.

Results

The chemically synthesized iron oxide nanoparticles with diameters less than 20 nm was tested by transmission electron microscopy (TEM) (Philips EM 208) to verify its uniform size and shape (Figure 1).

When infrared radiation passes through a sample, it absorbs certain wavelengths, causing vibrations such as elongation; shrinkage and warping of the chemical bonds in the material. Functional groups play an important role in this process. Therefore, FTIR method was used to detect changes in chemical bonds in the compounds Fe_2O_3, PEG and Fe_2O_3-PEG during infrared radiation (Figures 2 – 4).

To examine the cytotoxicity of iron oxide nanoparticles, MTT assay was performed. Royan B1 cells were treated on a medium containing different concentrations (10, 20, 30, 40, 50, and 60 µg/mL) of the iron oxide nanoparticles. Cell viability was determined at 12 and 24 hours after treatment. As it is shown in Figure 5, cell viability decreased in a concentration and time period dependent manner.

To investigate whether the nano Fe_2O_3 stimulated reactive oxygen species (ROS) generation in mES 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

Table 1. Embryoid body suspensions were treated with different amounts of iron oxide nanoparticles (NP) and 1 µM retinoic acid (RA). Group 1 received RA. Groups 2, 3, 4 were exposed to 10, 20, 30 µg/mL NP respectively. Groups 5, 6, 7 were exposed to RA (1 µM) and 10, 20, 30 µg/mL NP respectively. Group 8 as the control group had no treatment.

| No.	Treated groups
1	RA (1 µM)
2	Nanoparticles (10 µg/mL)
3	Nanoparticles (20 µg/mL)
4	Nanoparticles (30 µg/mL)
5	RA(1µM) + Nanoparticles (10 µg/mL)
6	RA(1µM) + Nanoparticles (20 µg/mL)
7	RA(1µM) + Nanoparticles (30 µg/mL)
8	Control (no treatment)
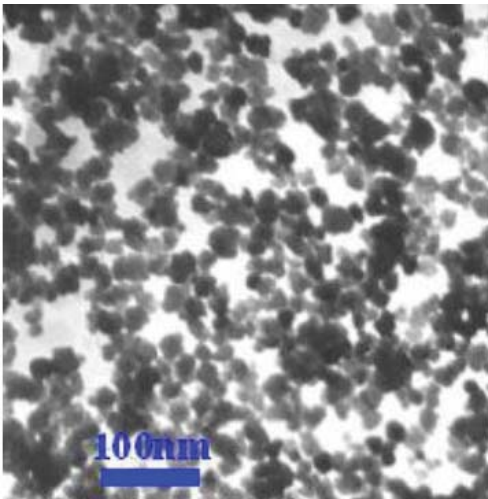
**Figure 1.** The TEM image of iron oxide nanoparticles. Particle size was less than 20 nanometers in diameter.
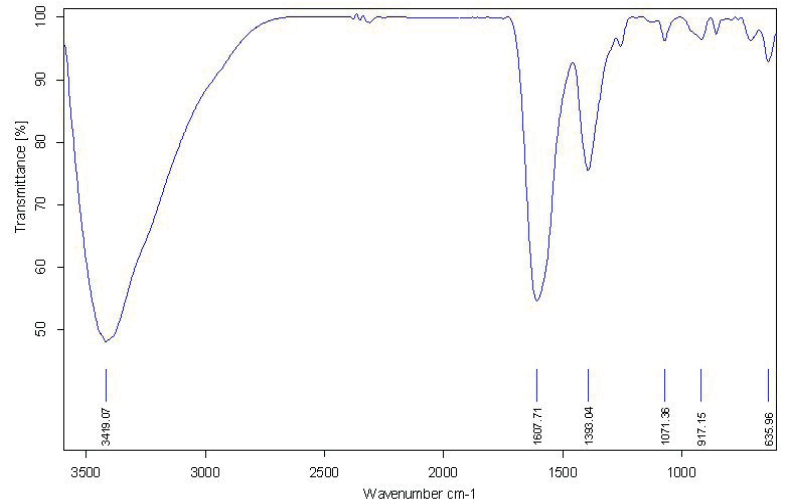
Figure 2. The Fe_2O_3 peaks. The peaks of Fe-O that are weak show their occurrence in regions 400 cm^{-1} and 600 cm^{-1} (are not shown). The peak begins in region 635 cm^{-1}.1607 cm^{-1} is related to structural changes in H-OH of water. 3419 cm^{-1} is also related to Stretching vibration of free OH in solution.
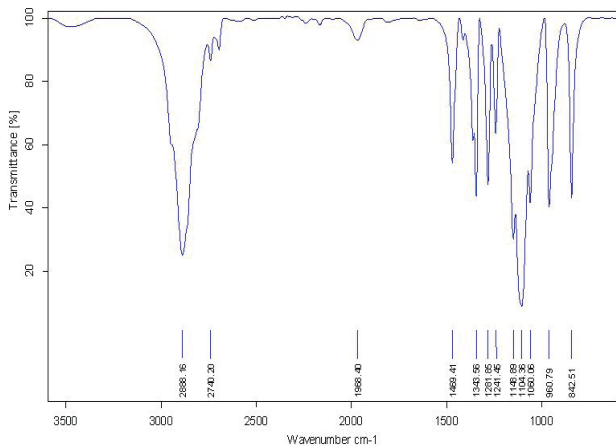

Figure 3. The PEG peaks. 842 cm^{-1} is related to Flexural vibrations of CH. 960 cm^{-1} is related to Stretching vibration of unpolymerized ethylene = CH minimal residues. 1105 cm^{-1} is related to strong Stretching vibration of C-O-C. 1469 cm^{-1} is related to strong Stretching vibration of CH2. 2888 cm^{-1} is related to strong Stretching vibration of CH.
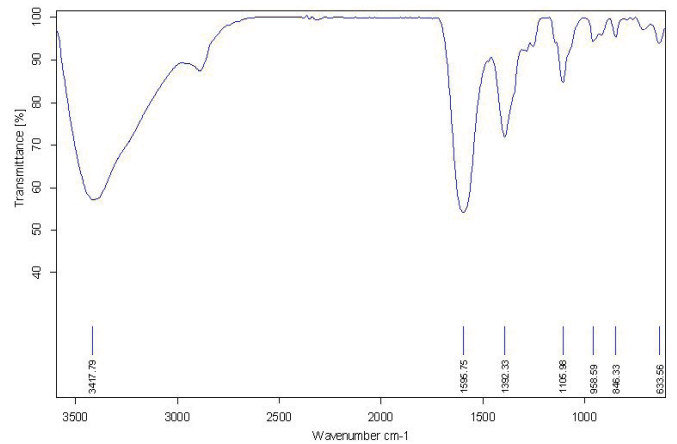

Figure 4. Peak of PEG-Fe_2O_3. CH peak (2888 cm^{-1}) and C-O-C peaks (1105 cm^{-1}) demonstrate the pegyylation of Fe_2O_3.
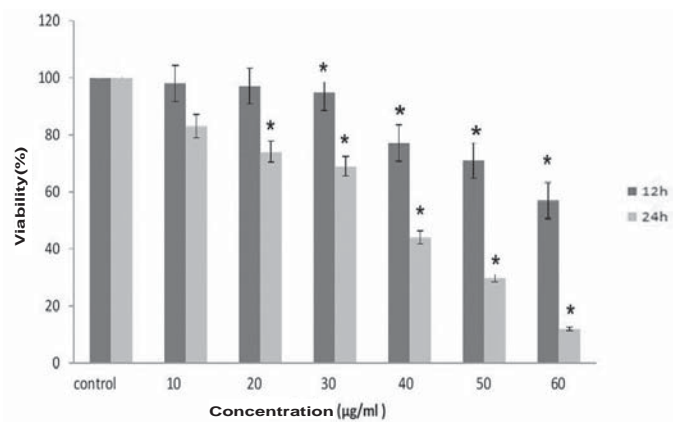
Figure 5. The cytotoxicity effect of iron oxide nanoparticles on mES cells treated with iron oxide nanoparticles (10, 20, 30, 40, 50 and 60 µg/mL) for 12 and 24 hours. After 12 and 24 hours cell viability assay was measured using MTT assay. The results were presented as mean ± SE of at least 3 independent experiments (*$P < 0.05$, compared to control).
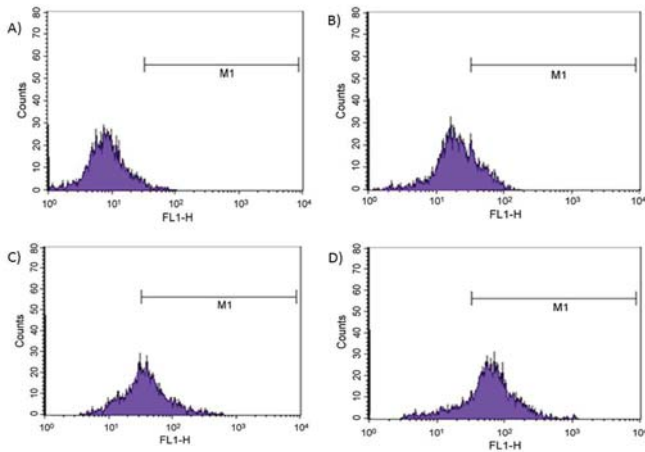
Figure 6. Measurement of ROS generation in mES cells. The cells were cultured with the nano-Fe_2O_3 at amounts of **A)** 0 µg/mL; **B)** 10 µg/mL; **C)** 20 µg/mL; **D)** 30 µg/mL for 12 hours. The generation of ROS was measured by flow cytometry. The level of ROS was increased in a dose-dependent manner. The corresponding histogram of flow cytometry was shown; **E)** n = 3, mean ± SEM, *statistically significant difference compared with controls (*$P < 0.05$, **$P < 0.01$).
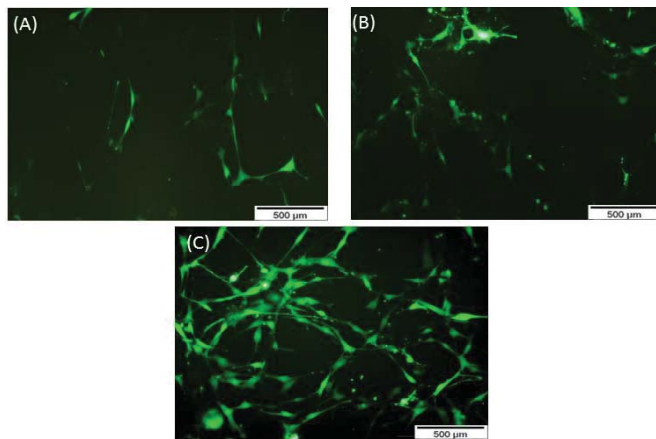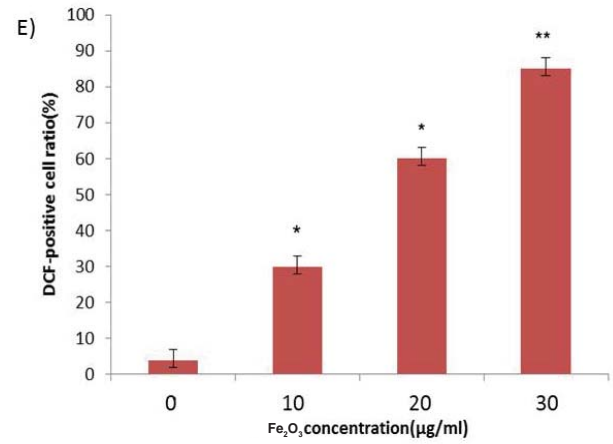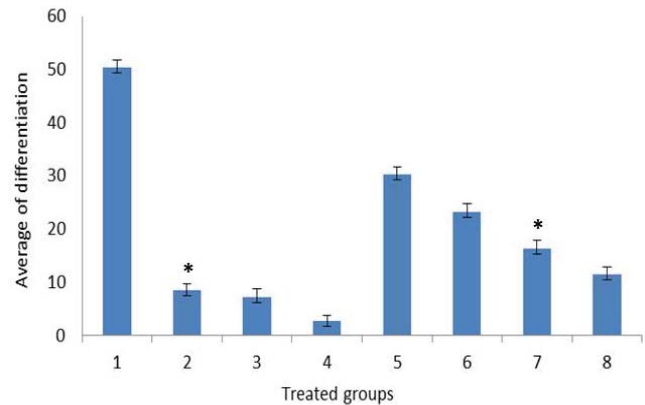

Figure 7. mESC immunofluorescenc for Tuj1 (b3-tubulin); **A)** cells treated with 10 µg/mL NP; **B)** cells treated with RA and10 µg/mL NP; **C)** cells treated with RA.

Figure 8. The effects of different amount of iron oxide nanoparticles and retinoic acid on neural differentiation of mES cells. Group 1 received RA. Groups 2, 3, 4 were exposed to 10, 20, 30 µg/mL NP respectively. Groups 5, 6, 7 were exposed to RA (1 µM) and 10, 20, 30 µg/mL NP respectively. Group 8 as the control group had no treatment. By adding NP a significant reduction in neuronal differentiation were observed (*$P < 0.05$, compared with control)

significantly. After the mES cells were exposed to the different amounts of 0, 10, 20 and 30 µg/mL nano Fe_2O_3 for 12 hours, the generation of the ROS was proved by the increased fluorescence intensity of oxidized DCF using flow cytometry. As shown in Figure 6, the ratio of DCF-positive cells was 4.25%, 27%, 55% and 85% at the amounts of 0, 10, 20 and 30 µg/mL nano Fe_2O_3, respectively. The results revealed that the level of ROS in mES cells, treated with the nano Fe_2O_3 suspension, was increased in a dose-dependent manner (Figure 6E).

Immunocytochemical analysis allowed for visualization of the neuronal differentiation using anti-b3-tubulin, a neural specific marker. In addition to mES cell viability, ability to respond to RA was also affected by treating with nanoparticles. Morphological analysis indicated that RA increased neuronal differentiation while following the addition of iron oxide nanoparticles a dramatic reduction in the ability of mESCs to generate neuritis was observed (Figure 7).

Discussion

Despite the increasing use of nanoparticles in the engineering, medicine and biology, their risks are still unclear. Due to their small size and high reactivity, nanoparticles can overcome the body barriers including blood- brain barrier. Therefore, the aim of this study was to evaluate the effects of iron oxide nanoparticle on the viability and neuronal differentiation of mouse embryonic stem cells.

To induce neural differentiation, RA was used as a potent inducer. Chemical induction is the most common and efficient way to induce differentiation of mouse embryonic stem cells into neural cells. After transferring embryoid bodies to non-adhesive bacterial dishes, EB suspensions were divided into 8 groups, receiving different amounts of nanoparticles (10, 20, 30 µg/mL), RA, or both. Differentiation was examined under phase contrast microscope and by using immunocytochemistry for b3tubulin. Group 1 (RA group) demonstrated the highest rate of neuronal differentiation as compared to control and nanoparticle groups. Morphological analysis indicated that following the addition of iron oxide nanoparticles a dramatic reduction in the ability of mESCs to generate neurite outgrowth was observed.

The results of this study demonstrated that iron oxide nanoparticles that were taken up by mES cells reduced RA-induced neurite outgrowth in a dose-dependent manner. In addition to mES cell ability to respond to RA, their viability was also affected by treating with nanoparticles. Exposure of cells to even moderate amounts of iron oxide nanoparticles adversely affected cell viability. These findings also indicate and confirm previous reports that the presence of intracellular Fe_2O_3 nanoparticle constructs can result in significant changes in cell behavior and viability.[13,14] The main mechanism of nanoparticles action is still unknown; however, *in vivo* and *in vitro* studies in different environments suggest that they are capable of producing ROS.[15,16] Measuring the level of ROS production in this study revealed an elevation of ROS by a dose dependent manner. The generation of ROS was proved by the increased fluorescence intensity of oxidized DCF. Elevation of ROS may have an effect on the concentration of intracellular calcium, activation of transcription factors, and creating changes in cytokine.[17] ROS can damage cells in various ways including damage to DNA, interfering with cell signaling pathways, and inducing changes in gene transcription.[18,19] The extent of damage caused by ROS depends not only on the type and amount but also the time and duration of exposure to ROS and external factors, such as temperature, pressure, oxygen, and deployment environment consisting of ions, proteins, and the amount removed on the ROS.[20] ROS production in the presence of nanoparticles can cause serious damages to the DNA. For example, chemical modification of histones or other proteins, that are involved in shaping the structure of DNA and open helical structure of DNA.[21–23] Although the mechanisms responsible for the reduced neurite outgrowth by iron oxide nanoparticles in mESC are still unclear, it has been presumed that Fe ions are released from iron oxide nanoparticles inside the cells, and these released ions reduce neurite outgrowth. Fe ions can stimulate free radical formation.[23] Fe is a strong oxidizing agent and stimulates the production of reactive hydroxyl radicals.[24] However, it is still possible that the iron oxide nanoparticles in this study may activate the extracellular receptor that in turn suppress the subsequent signal transduction for neurite outgrowth. Further studies are necessary to identify the key factors that affect the differentiation pathway by iron oxide nanoparticles.

In conclusions, according to the findings of this study, it can be concluded that in the presence of nanoparticles, viability and neural differentiation of mES cells (Royan B1 cell line) are reduced. Iron oxide nanoparticles that were taken up by mES cells are also reduced RA-induced neural differentiation in a dose-dependent manner. *In vivo* and *in vitro* studies in different environments suggest that iron oxide nanoparticles are capable of producing ROS. In this study, the level of ROS in mES cells, treated with the nano Fe_2O_3 suspension was increased in a dose-dependent manner. It seems that the main mechanism involved in the reduction of viability and neural differentiation was enhanced levels of ROS within the cells.

Acknowledgment

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