EFFECT OF IRON OXIDE NANOPARTICLES WITH ASCORBIC ACID ON NEURAL STEM CELLS

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Abstract

Cells labelled with iron oxide nanoparticles (ION) can be tracked by magnetic resonance imaging (MRI) in several applications. However, various studies demonstrated toxicity and oxidative stress induction associated with nanoparticles exposure. We analysed biologic effects after the exposure of two types of iron oxide nanoparticles (with and without an antioxidative agent; an ascorbic acid) on human neural stem cells. The labelled cells in gel phantoms were detected in MRI and they showed decreased relaxation rates in comparison with control. ION slightly decreased cell proliferation in comparison with unlabelled cells, which was dependent on concentration and presence of ascorbic acid. None of the nanoparticle type showed negative effect on cell viability and both demonstrated minor effect on reactive oxygen species (ROS) formation. Unfortunately, ascorbic acid bound to nanoparticles did not show any effect on ROS attenuation. Cells exposed to both types of nanoparticles showed increased positivity for a phosphorylated form of H2AX a marker of double strand breaks. We showed that ION in low concentrations do not affect cell viability, but have negative effect on cells on DNA level. Their potential use for oxidative stress reduction is dependent on the concentration of ascorbic acid bound to the nanoparticles and this should be further increased.

Keywords: Neural stem cells, nanoparticles, oxidative stress, ascorbic acid

1. INTRODUCTION

Stem cells became lately a promising tool for treatment of various injuries and diseases. To safely introduce stem cell therapy into clinical medicine, there is a need to understand the behaviour of transplanted cells and monitor the distribution of stem cells in a recipient organism [1]. Non-invasive imaging methods meet this requirement, especially magnetic resonance imaging has advantages over other technologies. It has very good resolution and it does not use ionizing radiation [2]. To view the cells on MRI, the cells need to be labelled with a contrast agent. Superparamagnetic IONs are widely used in experimental medicine, because they enhance the image contrast by decreasing the T_2 MRI signal. ION core is usually made of magnetite (Fe_3O_4) or maghemite (γ-Fe_2O_3), and the surface of superparamagnetic core is covered with a compatible coating to prevent agglomeration and to enhance biocompatibility [3]. Active molecules can be bound to the coating, which is used to tailor the nanoparticle for specific applications; e.g. active targeting [4]. Although IONs can be used in a variety of medical applications, such as targeted delivery of drugs or targeted destruction of tumour tissue by hyperthermia, exposure to IONs can be associated with significant toxic effects [5]. The toxicity of nanomaterials is associated mainly with generation of reactive oxygen species, which can further cause an oxidative stress. The latter can lead to the damage of macromolecules (DNA, lipids and proteins) [6]. In this study, we used γ-Fe_2O_3 nanoparticles, surface-modified with glucose and ascorbic acid, which possesses antioxidative properties and is an effective scavenger of reactive oxygen species. The effect of aforementioned particles on neural stem cells was investigated.

2. MATERIALS AND METHODS

2.1. Preparation and characterization of γ-Fe_2O_3 nanoparticles

γ-Fe_2O_3 nanoparticles were prepared according to the previously developed technique [7] and described in detail in Moskvin and Horák [8]. Maghemite nanoparticles were observed by a Tecnai Spirit G2 transmission electron microscope (TEM; FEI; Brno, Czech Republic) (**Figure 1**) Number-average diameter ($Dn=\Sigma Di/N$, Di is diameter of i-th particle and N is total number of the particles), weight-average diameter ($Dw=\Sigma Di\ 4/\Sigma Di\ 3$) and polydispersity index (PDI=Dw/Dn) were calculated from at least 300 individual particles on the micrographs using Atlas software (Tescan Digital Microscopy Imaging; Brno, Czech Republic). Modification of γ-Fe_2O_3 nanoparticle surface was conducted in three steps with (i)SiO_2, (ii) 5-(2,2-dimethyl-1,3-dioxolan-4-yl) -2,2-dimethyltetrahydrofuro[2,3-d] [1,3] dioxol-6-yl (3-(triethoxysilyl) propyl) carbamate (DFSC), and (iii) ascorbic acid (ASA).

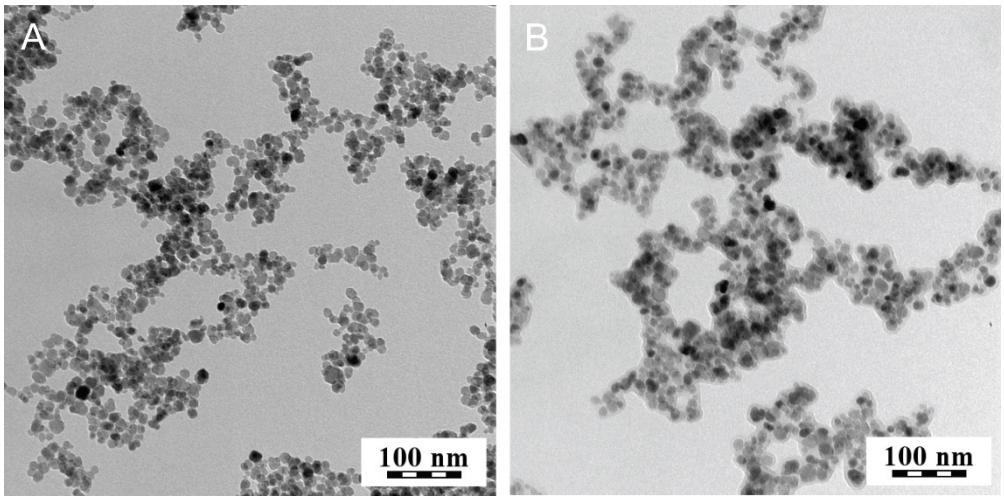

Figure 1 TEM micrographs of uncoated γ-Fe_2O_3 (A) and γ-Fe_2O_3&SiO_2-G-ASA (B)

2.2. Human foetal neural stem cells

A conditionally immortalized human foetal neural stem cell line SPC-01 was generated from 8-week-old human foetal spinal cord as described previously [9]. Briefly, cells were cultured in laminin coated tissue-culture flasks in medium supplemented with human serum albumin (0.03 %), human apo-transferrin (100 µg/ml), putrescine DiHCl (16.2 g/ml), human recombinant insulin (5 µg/ml), progesterone (60 ng/ml), L-glutamine (2 mM), sodium selenite (40 ng/ml), 4-OHT (100 nM), human EGF (20 ng/ml), human bFGF (10 ng/ml) and primocin 100 µg/ml, at 37°C and 5 % CO_2 in a humidified atmosphere

2.3. Cell labelling and proliferation analysis

Two types of nanoparticles (γ-Fe_2O_3&SiO_2 and γ-Fe_2O_3&SiO_2-G-ASA) were used for labelling of SPC-01, in various concentrations (5; 10; 15 or 30 µg Fe /ml in cultivation media) for 72 hours. Cell proliferation was monitored using the xCELLigence RTCA Instrument (ACEA Biosciences, USA). In the experiment, 40 000 of SPC-01 cells were seeded in the E plate wells coated with laminin. The cell proliferation in the presence of different concentrations of both types of nanoparticles was measured over a time period of 72 hours. Viability tests were performed with Alamar blue. Labelled or unlabelled SPC-01 cells were plated in 96-well plate (25 000 cells per well) and incubated for 24 hours. Before measurement 10 % solution of Alamar blue in cultivation medium was added to each well and incubated for 3 hours at 37°C. The fluorescence was measured using a Tecan-Spectra ELISA plate reader (Tecan) at 535/595nm. Statistical analyses were performed with t-test.

2.4. Measurement of oxidative stress

Labelled and unlabelled cells were treated with 15 mM H_2O_2 at 37°C for 30 min to induce oxidative stress. ROS was measured with Cellular Reactive Oxygen Species Detection Assay according to manufacturer instructions. To identify double strand brakes, antibody against phosphorylated H2AX was used. Labelled cells were washed in phosphate-buffered saline (0.1M PBS), and fixed in 4% paraformaldehyde in PBS for 30 min. Fluorescence images were taken with ZEISS AXIO Observer D1 microscope (Carl Zeiss, Weimar, Germany) and analysed with Image J program (NIH, USA).

2.5. MR relaxometry and imaging

T_2 relaxation time of the 4% gel phantoms containing the cells was measured on a Bruker Minispec MQ20 relaxometer (Bruker, Germany; 20 MHz, 21ºC) using a CPMG sequence with these parameters: 5 s recycle delay, 1 ms interpulse delay, 8 scans and 3000 points for fitting as described in detail in Jiráková et al. [10] Relaxivity r_2 was calculated as inverse relaxation time T_2 after deduction of the contribution of gel and unlabelled cells and related to cell concentration of the measured sample. T_2 weighted images of the 4% gel phantoms containing homogenously distributed cells were acquired on a 4.7 T Bruker Biospec scanner using a commercial resonator coil (Bruker, Biospin, Germany). A standard 2D rapid acquisition with relaxation enhancement (RARE) multispin echo sequence was used with the following parameters: repetition time (TR) = 3000 ms, echo time (TE) = 14 ms, spatial resolution = 137 × 137 μm^2, slice thickness = 0.5 mm, number of acquisitions = 1 and acquisition time = 9 min 36 s).

3. RESULTS AND DISCUSSION

3.1. Viability and proliferation

Cell proliferation was analysed in the presence of both types of nanoparticles: γ-Fe_2O_3&SiO_2 and with γ-Fe_2O_3&SiO_2-G-ASA for 72 hours. γ-Fe_2O_3&SiO_2 did not have any negative effect on cell proliferation in any used concentration (**Figure 2a**). In comparison, higher concentration 10 and 15 µg Fe/ml of Fe_2O_3&SiO_2-G-ASA resulted in slightly decreased cell proliferation however the decrease was not significant (**Figure 2b**). The effect of both types of SPION on cell viability was assessed by Alamar Blue. The viability of cells cultivated either with γ-Fe_2O_3&SiO_2 or Fe_2O_3&SiO_2-G-ASA were comparable to control unlabelled cells (**Figure 3**).

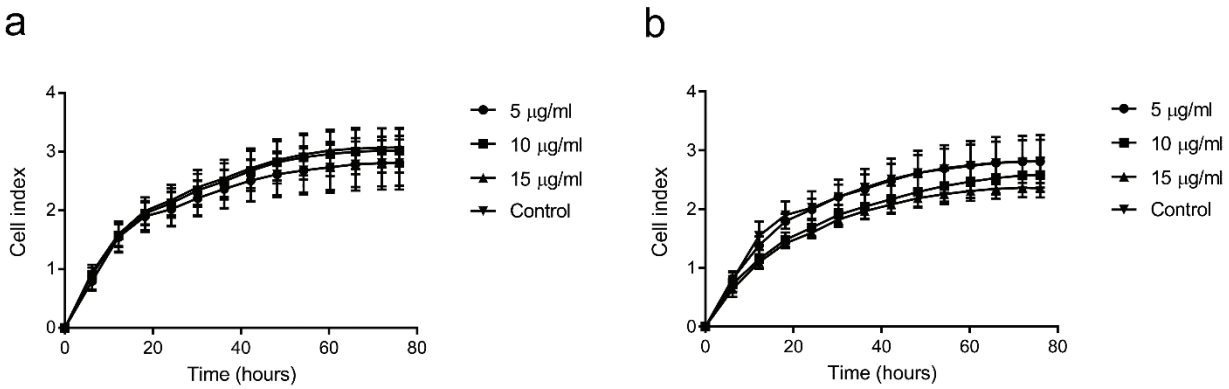

Figure 2 Proliferation profile of unlabelled control and labelled SPC-01cells. Proliferation curves of cells labelled with different concentrations of γ-Fe_2O_3&SiO_2 and unlabelled control (a) and with γ-Fe_2O_3&SiO_2-G-ASA and unlabelled control (b). Cell index represents cell viability, number, and morphology and adhesion degree. Graphs represent results from three independent experiments ± SEM

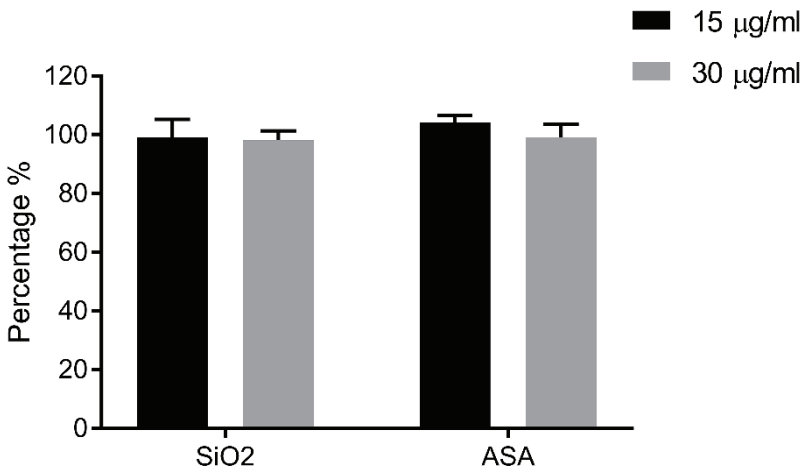

Figure 3 Viability of labelled cells. Alamar blue assay was performed after 72 hours incubation of SPC-01 cells with nanoparticles in concentrations 15 μg Fe/ml and 30 μg Fe/ml. The data are related to values of control unlabelled cells and are presented as a mean of three independent experiments ± SEM. SiO_2 - γ-Fe_2O_3&SiO_2; ASA - γ-Fe_2O_3&SiO_2-G-ASA.

3.2. Measurement of ROS production

Oxidative stress is one of the most common toxic effect connected with nanoparticles exposure. To monitor the levels of oxidative stress, the ROS level was measured in γ-Fe_2O_3&SiO_2 and γ-Fe_2O_3&SiO_2-G-ASA treated cells. As a positive control were used cells treated with 15 mM H_2O_2. Results do not show increased ROS level in cells labelled with either of nanoparticle type in comparison with control (**Figure 4**). The presence of H_2O_2 increased the ROS level either in control or in labelled cells. The difference was significant between control and labelled cells treated with H_2O_2 ($p < 0.05$). Staining for the presence of phosphorylated form of H2AX a marker for DNA double strand breaks showed positivity in cells after treatment with H_2O_2 and in cells labelled with both types of nanoparticles (**Figure 5**).

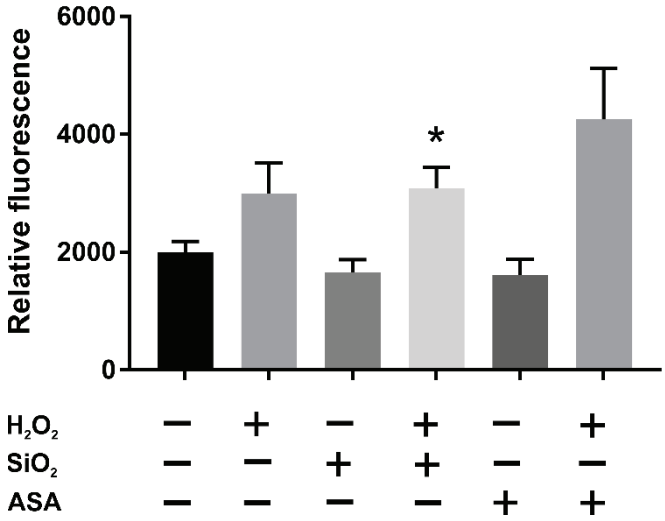

Figure 4 Production of reactive oxygen species. Oxidative stress was measured in SPC-01 cells treated with either γ-Fe_2O_3&SiO_2 (SiO_2) or γ-Fe_2O_3&SiO_2-G-ASA (ASA) at concentration 15 μg Fe/ml for 72 hours and H_2O_2 15 mM. Data are presented as mean of relative fluorescence ± SEM. *$p < 0.05$ (control (black bar) vs. SiO_2 treated with H_2O_2)

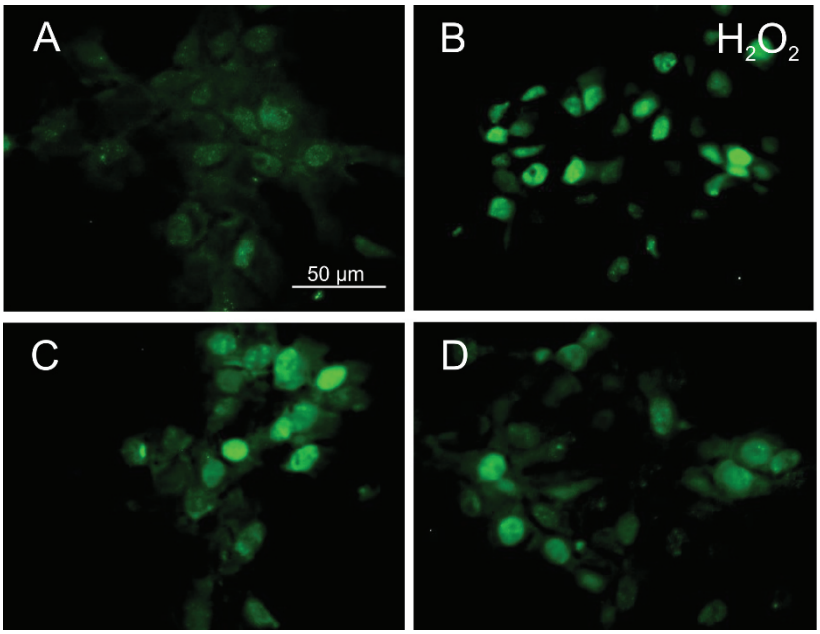

Figure 5 Immunocytochemistry of H2AX in SPC-01. Control unlabelled cells (A) and treated with H_2O_2 (B), cells labelled with γ-Fe_2O_3&SiO_2-G; 15 µg Fe/ml (C), or cells labelled with γ-Fe_2O_3&SiO_2-G-ASA; 15 µg Fe/ml (D)

3.3. MR relaxometry and imaging

To test the detectability by MR imaging, SPC-01 labelled for 72 hours with different concentrations γ-Fe_2O_3&SiO_2 and γ-Fe_2O_3&SiO_2-G-ASA nanoparticles were immobilized in 4% gelatin phantom. Relaxometry of both types of nanoparticles were comparable at concentration 15 µg Fe/ml (**Table 1**). On the other hand, relaxometry revealed approximately twice higher r_2 values in γ-Fe_2O_3&SiO_2-G-ASA labelled cells in comparison with γ-Fe_2O_3&SiO_2 labelled cells at concentration 30 µg Fe/ml. Labelled cells were detected by MR imaging as hypointense spots and data corresponded with relaxometry values (**Figure 6**). Larger and more hypointense area was observed for cells labelled with γ-Fe_2O_3&SiO_2-G-ASA at concentration 30 µg Fe/ml than in γ-Fe_2O_3&SiO_2 labelled cells.

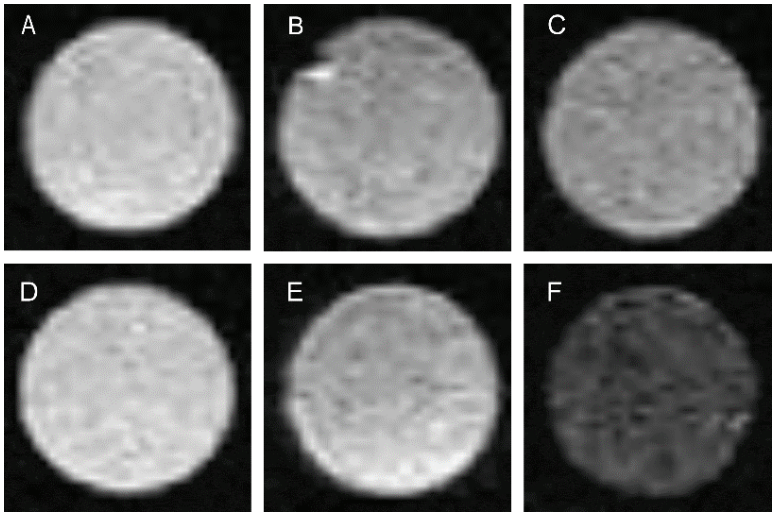

Figure 6 MR images of gel phantoms. Gel phantoms without cells (A), containing unlabelled cells (D), or SPC-01 labelled with γ-Fe_2O_3&SiO_2-G; 15 µg Fe/ml (B); 30 µg Fe/ml (C) or γ-Fe_2O_3&SiO_2-G-ASA; 15 µg Fe/ml (E); 30 µg Fe/ml (F)

Table 1 Relaxivity r_2 *in vitro* of labelled cells

$r_2[s^{-1}/$ mil cells/ml] ± SD		
Nanoparticles	15 Fe [μg/ml]	30 Fe [μg/ml]
γ-Fe_2O_3&SiO_2	0.28 ± 5.005	0.40 ± 0.010
Fe_2O_3&SiO_2-G-ASA	0.25 ± 0.005	0.86 ± 0.006

4. CONCLUSION

We studied the biological effect of γ-Fe_2O_3&SiO_2 nanoparticles with or without ASA coating on neural stem cells. Cell proliferation analysis revealed only minor decrease in proliferation rate of labelled cells. Neither type of nanoparticle in any used concentration showed significant negative effect on cell viability or increased ROS production in neural stem cells. Negative effect of nanoparticle exposure was revealed on DNA level as showed the presence of H2AX. γ-Fe_2O_3&SiO_2 labelling is suitable for MR imaging; the relaxivity rates and number of hypointense spots were proportional to used iron concentration. The antioxidative activity of nanoparticles would be valuable but needs detailed investigation especially according to effective concentration of antioxidant compound.

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