Original Article

Evaluating the effect of ultrasmall superparamagnetic iron oxide nanoparticles for a long-term magnetic cell labeling

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ABSTRACT

In order to evaluate the long-term viability, the iron content stability, and the labeling efficiency of mammalian cells using magnetic cell labeling; dextran-coated ultrasmall superparamagnetic iron oxide (USPIOs) nanoparticles with plain surfaces having a hydrodynamic size of 25 nm were used for this study. Tests were carried out in four groups each containing 5 flasks of 5.5×10^6 AD-293 embryonic kidney cells. The cell lines were incubated for 24 h using four different iron concentrations with and without protamine sulfate (Pro), washed with phosphate-buffered saline (PBS) and centrifuged three times to remove the unbounded USPIOs. Cell viability was also verified using USPIOs. There were no significant differences in the cell viability between the control group of cells and those groups with iron uptake at the specified iron concentrations. The average iron uptake ratio compared to that of the control group was (114 ± 1). The magnetic resonance images (MRI) at post-labeling day 1 and day 21 showed (75 ± 4)% and (22 ± 5)% signal decrements compared to that of the control, respectively. The Perl's Prussian blue test showed that 98% of the cells were labeled, and the iron concentration within the media did not affect the cell iron uptake. Magnetic cellular labeling with the USPIO-Pro complex had no short or medium term (3 weeks) toxic effects on AD-293 embryonic kidney cells.

Key words: Cell labeling, cell viability, magnetic resonance images, protamine sulfate, ultrasmall superparamagnetic iron oxide

Introduction

Previous studies of *in vivo* cell trafficking were mostly dependent on the use of radionuclide labels such as indium-111,[1] but there were concerns about the use of the radioisotopes for the labeling of long-living cells such as lymphocytes. In addition, there are also concerns about long-term changes in cells which recirculate.[2]

Recent studies indicate that there are a wide range of applications for magnetic resonance imaging (MRI) in molecular and cellular imaging studies.[3-5] There is an increasing interest in using MRI to observe the *in vivo* behavior of stem and other cells labeled with ultra small superparamagnetic iron oxide (USPIO) nanoparticles. The USPIO nanoparticles are emerging as an ideal probe for noninvasive cell tracking. Appropriate sized nanoparticles allow efficient particle-cell interaction. The magnetic properties of the nanoparticles permit accurate, noninvasive and real-time cell tracking.[6] In particular, the prolonged cellular retention time of these particles provides a large observation temporal window, and therefore makes it possible for long-term *in vivo* cell tracking. However, the ability to monitor cell survival, cell migration, and its differentiation are additional properties that are required for the success of cell based therapies.

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The use of USPIO has been limited due to its low intracellular labeling efficiency; therefore new labeling strategies are needed.[7-9] Previous studies showed a promising effect on the on the cell labeling efficiency, due to the addition of protamine sulfate.[10] It is a low-molecular-weight (~4 kDa), naturally occurring polycationic peptide that is approved by the Food and Drug Administration (FDA) as an antidote to heparin anticoagulation.[11,12] Protamine sulfate is well-tolerated by cells, with a high therapeutic window of more than 50 mg/ml.[13]

Although USPIO agents were developed a decade ago, there is still lack of data on cell tracking and long-term follow up of cells after cell labeling. The aim of this study is to evaluate the long-term viability, the iron content stability, and the labeling efficiency of mammalian cells when using the USPIO nanoparticles with plain surfaces combined with protamine sulfate (USPIO-Pro) for magnetic cell labeling.

Materials and Methods

Nanoparticles characterization

The nanoparticles were purchased from Micromod GmBH (Rostock, Germany) with lot number 79-00-201.[2,3] The characteristics of the colloidal suspension were examined by different methods. The size and morphology of dextran-coated ultrasmall superparamagnetic iron oxide (USPIOs) nanoparticles were investigated by transmission electron microscope (TEM, Philips CM100) and field emission scanning electron microscope (FE-SEM, Hitachi 4160). After the micrographs were obtained, image analysis was performed by the Clemex Vision PE 4 software. The hydrodynamic diameter of the suspended nanoparticles was determined by dynamic light scattering measurements (DLS, Malvern Instruments) using 632 nm wavelength laser. A turbidimeter (Martini Instrument MI415) apparatus was used to evaluate the colloidal stability of suspensions qualitatively at different time points, from 1 h to 45 days, on the basis of light absorbance of USPIOs. In all cases, the suspensions were diluted to prepare such a sufficient concentration of nanoparticle for allowing light transmission. The magnetic behavior of solid nanoparticles was obtained by a vibrating sample magnetometer technique (VSM, Maghnetic Daghigh Kavir Co). In this process, the samples were magnetized in 0.06 (Tesla) external magnetic field and the major magnetic parameters like saturation magnetization (M_s) and coercivity (H_c) values were calculated. In addition, atomic absorption spectroscopy (AAS, GBC Avanta) and inductively coupled plasma atomic emission spectroscopy [Inductively Coupled Plasma ICP-AES, Varian-Liberty 150 AX Turbo] were performed to determine iron concentration of the suspensions.

Preparation of ultrasmall superparamagnetic iron oxide-Protamine sulfate (USPIO-Pro) complex

The commercially available protamine sulfate (American Pharmaceuticals Partner, Schaumburg, IL) supplied at 10 mg/ml, was prepared as a fresh stock and mixed with magnetic nanoparticles having 2.2 mg Fe/ml with a ratio of 100:8 (USPIO: Pro), respectively.

Cell culture

Cell lines were obtained from the National Cell Bank of Iran (NCBI, Pasteur Institute of Iran, Tehran). The AD-293 embryonic kidney cells were cultured into a sterile plastic culture flask (Nunc, Denmark). The culture flask was filled with a thin layer of complete culture medium, DMEM, (Gibco, USA) with 10% fetal calf serum (FCS) supplemented with 100 U/ml penicillin and 100 μg/ml streptomycin (Sigma, UK) and incubated in a CO_2 incubator at 5% CO_2 and 37°C with saturated humidity. Following 24 h-48 h incubation time, the cells were detached by gently pipetting[3,14] when cells reached 80-90% confluency. Cell viability was assessed by the trypan blue (Sigma, UK) dye exclusion method. In this process, 1.5×10^6 cells were placed in a 75 cm^2 flask the day before labeling.

Cell labeling process

The tests were carried out in four groups, each containing 5 flasks with 5.5×10^6 AD-293 embryonic kidney cells. USPIO-Pro complex was incubated with AD-293 embryonic kidney cell lines for 24 h with four different iron concentrations (0 mg/ml, 0.25 mg/ml, 0.5 mg/ml, and 0.7 mg/ml). The cells were detached by gently pipetting after 24 h post-labeling with USPIO-Pro complex. Initially, the cells were washed with phosphate-buffered saline (PBS) and centrifuged at 900 rpm three times to remove the unbounded USPIO-Pro complex. Iron uptake was assessed using Perl's Prussian blue test, *in vitro* MRI imaging, and ICP-AES analysis. The iron concentration in the media were assessed at different time points (1 day to 21 days post-labeling) with the same number of cells and by counting the cells with trypan blue staining.

Iron concentration measurement

The cells were stained with routine hematoxylin-eosin (for cell morphology), Perl's Prussian blue (for iron uptake), and Turnbull's reaction (for particle detection). The Perl's Prussian blue reaction stains mainly Fe^{3+} ions, whereas the Turnbull's reaction can detect Fe^{2+} ions. For a short period, the prepared slides were immersed in 2% solution of potassium ferrocyanide and hydrochloric acid (HCl) at equal volumes (1:1) for 20 min for Perl's Prussian blue staining. After three washings of the slides in distilled water (dH_2O), counter staining was done by incubating the slides in neutral red solution for 10 min before two final washings in dH_2O. Turnbull's reaction differs from the Pearl's Prussian blue staining only in the mixture of the

solution, where 20% potassium ferrocyanide and 1% HCl are mixed at equal volumes. After air-drying the slides, they were covered with a cover slip mounted on glycerin-gelatin treated glass slides.

Determination of mean iron concentration per cell

After labeling, cells were washed three times with PBS. Specific numbers of unlabeled and labeled cells were then collected, centrifuged, and counted with trypan blue. For the quantification of average iron concentration per cell, the same amount of each group was suspended in 1 ml microtubes for MRI. Later, the cell suspensions with known cell density were dried overnight at 110°C and then completely digested in a mixture (500 μl) of perchloric and nitric acid at a ratio of 3:1. The samples were digested for at least 6 h digestion in 50% v/v HCl at 60°C using a heating block[10] and sent for ICP-AES analysis. Iron concentration in samples was calculated from a standard curve that was derived from calibration standards of ferumoxides containing 0 mM Fe to 1 mM Fe in the same acid mixture. Iron concentration was expressed as an average picogram iron/cell. There were at least three samples for each condition at different time points.

Determination of labeling efficiency

The labeling efficiency was determined by manual counting of Perl's Prussian blue stained and unstained cells using a Zeiss microscope (Axioplan Imaging II, Zeiss, Oberkochen, Germany) at ×100 magnifications. The images were processed by Adobe Photoshop 7.0 (San Jose, CA). Cells were considered PB positive if the intracytoplasmic blue or brown (DAB enhanced) granules could be detected. The percentage of labeled cells was determined from the average of 5 to 10 high-powered fields.

Cell viability assay

The viability of cells and their numbers were evaluated by trypan blue staining. At each passage, the cell viability was checked by adding a vital stain to a portion of the cell suspension (100 ml cell suspension: 100 ml trypan blue, 0.4%). The cells were left at room temperature for three minutes. Using a Neubauer improved hemocytometer, the dead and the living cells were hand counted (three different aliquots per digested carrier, each counted three times) and the cell viability was evaluated.[15] The cellular uptake of USPIOs at each stage was determined by measuring the iron content using ICP-AES analysis and Perl's Prussian blue staining.

Magnetic resonance images measurements

MRI of samples (in test tubes) was performed using a 1.5-T MR scanner (Signa, GE Medical Systems, Milwaukee, WI, USA) and a standard circularly polarized head coil (Clinical MR Solutions, Brookfield, WI, USA). All probes were placed in a water-containing plastic container [Figure 1] at room temperature (25°C) to avoid susceptibility artifacts from the surrounding air in the scans.

Longitudinal relaxation time (T1)- and spin-spin or transverse relaxation time (T2)-weighted spin echo (SE) images were acquired using variable repetition time (TR) and echo (TE) times of TR/TE = 256 ms/16ms, and TR/TE = 3,000 ms/64 ms, and then analyzed qualitatively. Initially, the signal intensities of all test tubes with contrast medium at different iron concentrations were assessed visually. For quantitative data analysis, the images were transferred to a local workstation, and the T1 and T2 maps were calculated assuming a monoexponential signal decay. T1 maps were calculated using four SE images with a fixed TE of 11 ms. The TR values of 4,000 ms, 2,000 ms, 1,000 ms, 500 ms, and 250 ms were calculated using a nonlinear function least-square curve fitting on a pixel-by-pixel basis. The signal intensity for each pixel as a function of time was expressed as follows: $SI_{(pixel\ xy)}(TR) \approx S_{o\ (pixel\ xy)}[1-\exp(-TR/T1_{(pixel\ xy)})]$. T2 maps were calculated accordingly from four SE images with a fixed TR of 4,000 ms and TE values of 12 ms, 24 ms, 36 ms, and 48 ms on the 1.5-T MR scanner. The signal intensity for each pixel as a function of time was expressed as follows: $SI_{(pixel\ xy)}(TE) \approx S_{o\ (pixel\ xy)}[1-\exp(-TE/T2_{(pixel\ xy)}]$. Care was taken to analyze only data points with signal intensities significantly above the noise level.

T2*-weighted images were obtained with a TR of 300 ms, TE of 12 ms, and a flip angle of 15°. All sequences were acquired with a field of view of 160 × 160 mm, a matrix of 256 × 196 pixels, and slide thickness of 3 mm.

In order to see the use of protamine sulfate labeling efficiency, we used 1/10th and 1/100th of the labeled cells after 3 weeks post-labeling into the microtubes (i.e., 5.5×10^5 and 5.5×10^4, respectively) and tried to detect them in T2*-weighted images.

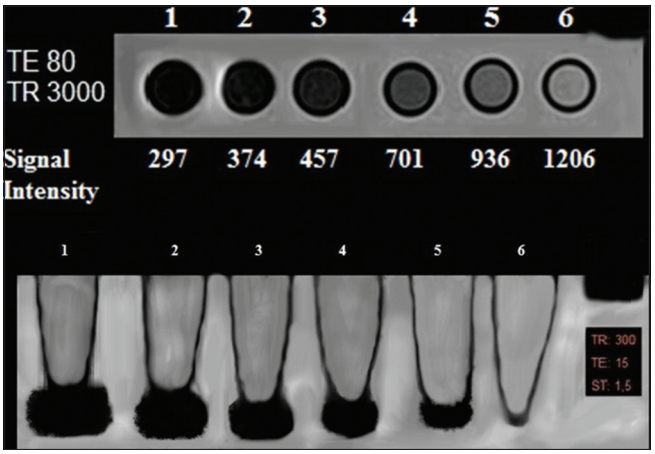

Figure 1: MRI images of labeled cells with USPIO-Pro Complex. (a) T2-weighted images of labeled cells. (1): 1 day, (2): 3 days, (3): 1 week, (4): 2 week, (5): 3 week post-labeling and (6): control group. All sample cells were labeled with 0.7 mg/ml of USPIO-Pro complex. Images were obtained at TR = 3000 ms and TE = 80 ms and (b) representative axial T2*-weighted images of the labeled samples with USPIO-Pro complex. T2* images were obtained at TR = 300, TE = 15 ms, and flip angle = 15°

Results

Figure 2 shows the MH curve of USPIO using VSM measurement. The saturation magnetization is about 69 Am^2/kg iron ($H > 795.8$ A/m).

The ICP results, viability, percentage of cell labeling with nanoparticles and the signal changes are shown in Table 1.

Figure 3 and Figure 4 show the size information of the prepared USPIOs. As demonstrated in Figure 3, the size distribution of the nanoparticles is fairly narrow. Therefore, it may be attributed to ultrasonic irradiation and the use of the polymers as a biocapping agent. The agglomerated morphology of the observed nanoparticles in TEM micrographs arise from the drying process in consequence of their high specific surface area and surface energy. However, this defect can be resolved using spin coating techniques for drying, or alternatively, using FE-SEM.

The DLS histogram of the colloidal suspension of nanoparticles is shown in Figure 5. There is no significant difference between the hydrodynamic size of nanoparticles obtained from DLS and the average size calculated by FE-SEM. This is due to a minor hydrated layer on the surface of coated nanoparticles, which is attributed to various factors such as spatial conformation and surface tension (energy) of the coated material.

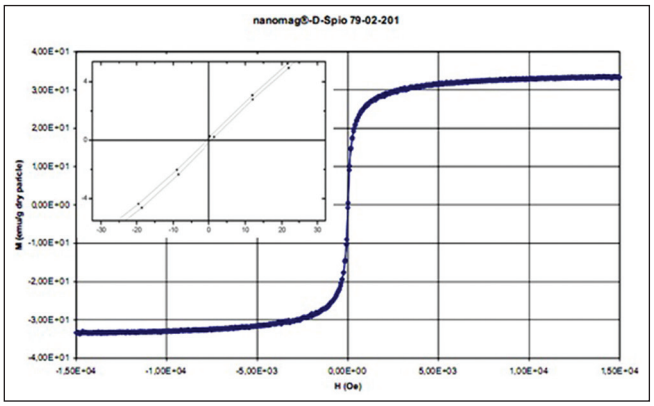

Figure 2: VSM measurement of USPIOs. Coercivity values can be seen in focused intersects on magnetization curve

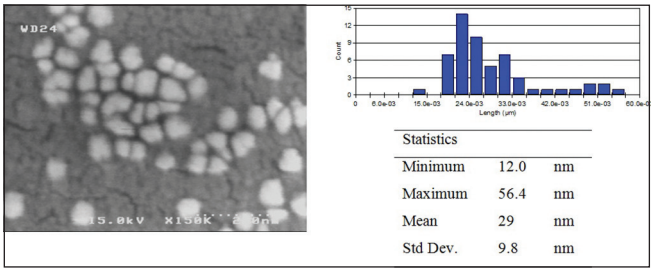

Figure 4: Field Emission Scanning Electron Microscopy FE-SEM micrographs of USPIOs (Clemex image processing for related micrographs)

The relaxometry results are shown in Figure 6. The r1 and r2 values are in accordance with our previous study.[3]

The results of Perl's Prussian blue staining of the AD-293 embryonic kidney cells labeled with USPIO-Pro complex are shown in Figure 7. The results were obtained after 1 day, 3 days, 7 days, 14 days, and 21 days post-labeling using a labeling process where the cells were incubated with 0.7 mg/ml of USPIO-Pro complex.

Discussion

There are a broad range of applications for MRI in cellular imaging studies. One of the promising contrast agents with very high relaxivity are the USPIOs. These particles have a strong effect on MRI signal intensities if sufficient amount can be labeled to the target cells and retained for a long-term during the cell tracking process.[17]

In this study, four different concentrations of USPIO-Pro complex (0 mg/ml, 0.25 mg/ml, 0.5 mg/ml, and 0.7 mg/ml) were used. As no significant differences were found among these four groups in terms of iron uptake [Table 2], all the viability and imaging results were performed on the samples labeled with 0.7 mg/ml of USPIO-Pro complex within 3 weeks post-labeling. The proliferation and the

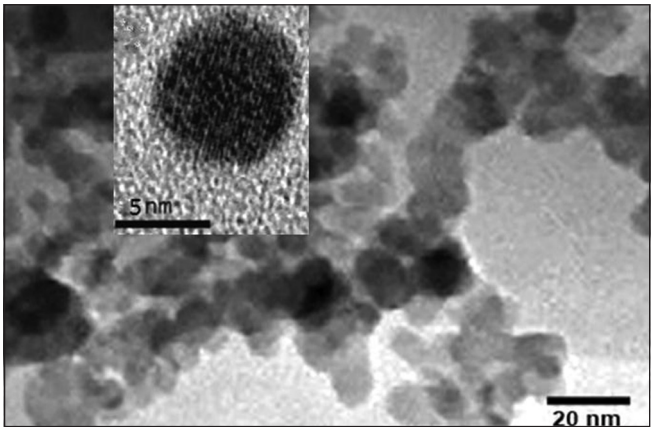

Figure 3: TEM of a USPIO sample reveals the crystal core size of the particles to be about 5 nm[16]

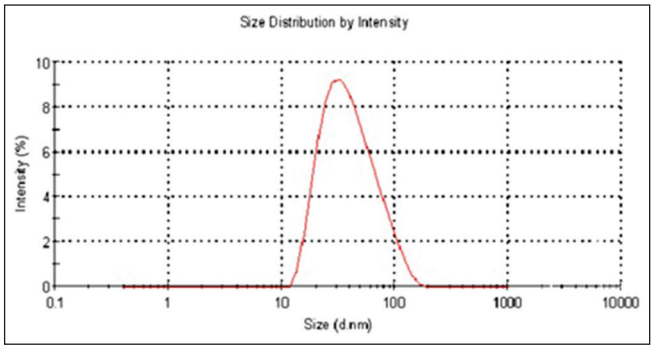

Figure 5: DLS histogram of USPIOs indicating their hydrodynamic magnitudes of about 25 nm

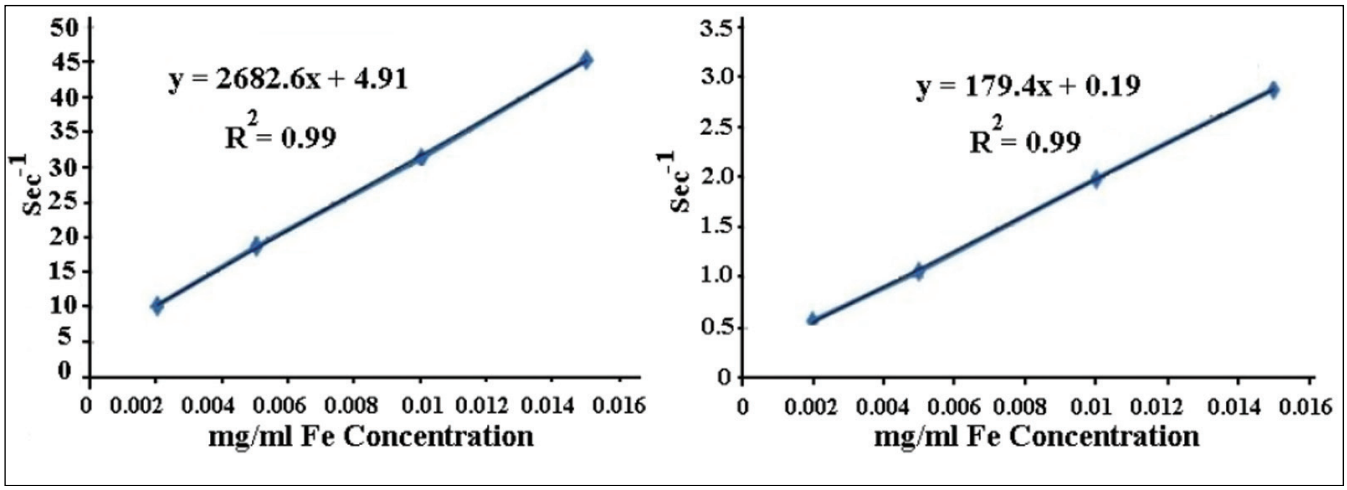

Figure 6: Relaxometry tests show that r2 of USPIOs is 2682.6 ml/mg.s or 11.56 (mMol^{-}1s^{-}1) and r1 value is 0.77 (mMol^{-}1s^{-}1)

Table 1: Viability, iron concentration (ICP results), % of iron labeled by Perl's Prussian blue staining and signal changes by magnetic resonance images imaging for the labeled cells with USPIO-Pro complex. Sample properties are defined in Figure 1

Sample number	*Viability (%)*	*Post-labeling days*	*Iron content (ppm or μg/ml)*	*Perl's prussian blue (%)*	*Iron content per cell (pg/cell)*	*Number of particles per cell*	*% Signal loss in T2-weighted*
0	>90	1*[1]	3.7±0.3	25±3	0.67	53	−10±3
1	>90	1	62.5±1.1	98±2	11.36	909	−75±4
2	>90	3	54.3±0.9	89±5	9.87	789	−69±4
3	>90	7	49.5±0.8	50±7	9.00	720	−62±4
4	>87	14	25.3±0.5	31±5	4.60	368	−42±3
5	>85	21	10.8±0.4	25±4	1.26	157	−22±5
6	>90	Control	0.6±0.2	0	0.1	NA	0

*[1]Labeled with USPIOs without protamine sulfate. There are 8×e[13] particle per mg Iron, and 5.5×e[6] cells in each group

Table 2: Viability, iron concentration (ICP results) among different iron concentration incubation

Sample number	*Viability (%)*	*Post-labeling days with 0.25 mg/ml, 0.5 mg/ml, and 0.7 mg/ml USPIO-Pro*	*Iron content (ppm or μg/ml) respectively*
0	>90	1*[2]	3.7±0.8, 3.7±0.2, 3.7±0.3
1	>90	1	62.7±0.8, 62.1±1.2, 62.5±1.1
2	>90	3	53.9±1.2, 54.1±0.5, 54.3±0.9
3	>90	7	49.8±0.2, 49.1±0.5, 49.5±0.8
4	>87	14	25.1±0.8, 25.7±0.1, 25.3±0.5
5	>85	21	10.9±1.0, 10.6±0.1, 10.8±0.4
6	>90	Control	0.55±0.19

*[2]Labeled with USPIOs without protamine sulfate. There are 8×e[13] particle per mg Iron, and 5.5×e[6] cells in each group

cell viability did not change within this period.

In our study, the iron uptake of the cells after one day post-labeling with USPIOs without protamine sulfate showed less iron contents than those with USPIO-Pro complex even after 3 weeks post-labeling. These results are different from the labeling efficiency of Sun *et al.*[18] in which they found the iron content of the labeled cell to be less than that of the control group after 15 days post-labeling. This disagreement clearly shows the importance of using protamine sulfate and its effects on cell labeling. The use of protamine sulfate in cell labeling was studied previously by a number of researchers,[10,19] but not for the medium or long-term follow-up of the labeled cells.

This viability study was consistent with the results of Arbab *et al.*[10] who concluded that ferumoxides-protamine sulfate complexes labeled cells demonstrated no short or long-term toxicity up to 8 days post-labeling. They also showed no change in differentiation capacity of the stem cells and no change in their phenotype when compared with that of the unlabeled cells. Our results are also consistent with previous studies [10,20-22] which showed that cell proliferation was not affected by plain USPIO when compared to control cells.

One of the limitations of this study was that they did not have access to Bio-TEM to check whether the USPIOs

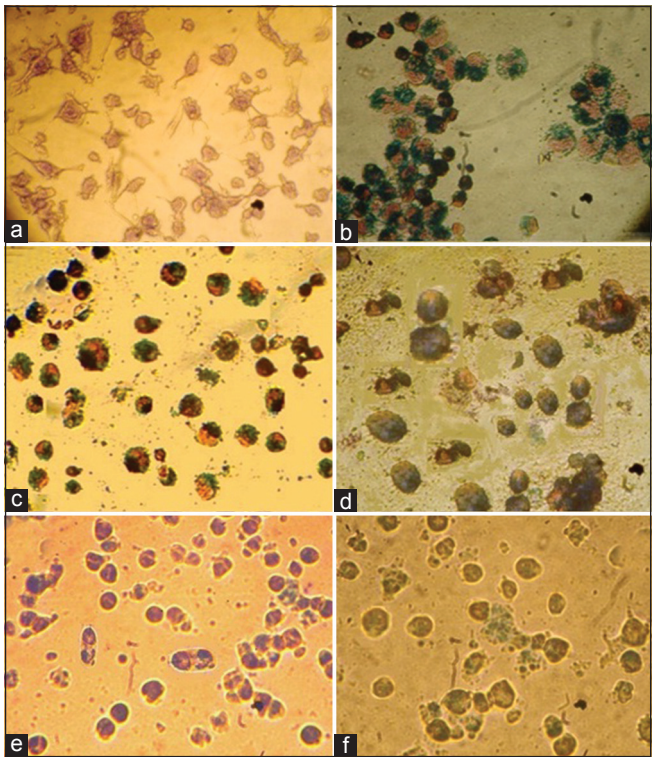

Figure 7: A sample result of Perl's Prussian blue stained USPIO-Pro-labeled AD-293 cells. (a) Unlabeled cells, (b) 1 day, (c) 3 days, (d) 1 week, (e) 2 weeks, and (f) 3 weeks post-labeling, using ×250 magnifications. All labeled cells were incubated with 0.7 mg/ml of USPIO-Pro complex

are attached to the surface of the cells or whether they are present inside the cells. However, by multiple focusing of Perl's Prussian blue images, they concluded that the particles were inside the cells [Figure 7e].

Numerous studies have shown that the cellular uptake is dependent on particle size, by which the maximum uptakes occur for larger crystal sizes.[18] Sun *et al.*[18] concluded that mesenchymal fibroblast and epithelial HEPG2 cell lines accumulated SPIO (~60 nm) more efficiently than USPIO (~30 nm) indicating that SPIO is better suited for cell labeling.[18] These results suggest that by using SPIO and manipulating protamine sulfate we may have better iron uptake and can trace the cells for a longer duration. However, further studies and comparison are required.

Conclusion

In this study, USPIO-Pro complex was used to effectively label the AD-293 embryonic kidney cell lines with no short or long-term (3 weeks post-labeling) effects on cell viability. Results in this study showed an excellent contrast between the labeled and control cells. Even when a very low concentration of iron is used, a good contrast can be detected between the labeled and unlabeled cells after 3 weeks.

Magnetic labeling of cells with USPIO-Pro complex holds promise for monitoring the temporal and spatial migration of stem cells into tissues. It is therefore possible to improve the development of cell tracking strategies for the repair or regeneration of tissues and other cell therapies.

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