

Original Article

Evaluation of Optimal Combination of Commercially Available Superparamagnetic Iron Oxide Nanoparticles and Transfection Agents for Labelling of Human Mesenchymal Stem Cells

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Purpose : To determine the optimal combination of commercially available superparamagnetic iron oxide (SPIO) nanoparticles with transfection agents (TA).

Materials and Methods: Protamine sulfate (Pro) and poly-L-lysine (PLL) were incubated with ferumoxide and ferucarbotran in human mesenchymal stem cells at various concentrations, and cellular viability were evaluated. Cellular iron uptake was qualitatively and quantitatively evaluated. Cell visibility was assessed via MR imaging and the T2-relaxation time was calculated.

Results: The cellular viabilities with ferucarbotran were more significantly decreased than those with ferumoxide ($p < 0.05$). Iron uptake with ferumoxide was significantly higher than that for those with ferucarbotran. The T2-relaxation time was observed to be shorter with ferumoxide in comparison to those with ferucarbotran ($p < 0.05$). Ferumoxide at a concentration of 25 $\mu\text{g/ml}$ in combination with either Pro or PLL at a concentration of 3.0 $\mu\text{g/ml}$ did not adversely impact cell viability, maximized iron uptake, and exhibited a lower T2-relaxation time in comparison to other combinations.

Conclusion: Stem cells with ferumoxide exhibited a higher cellular viability and iron uptake in comparison to ferucarbotran-treated stem cells. A 25 $\mu\text{g/ml}$ of ferumoxide with a 3.0 $\mu\text{g/ml}$ of TA is sufficient to label mesenchymal stem cells.

Index words : Cell labeling · Contrast media · Experimental studies · Iron · Magnetic resonance (MR)

INTRODUCTION

Advances in stem cell transplant therapies have progressed and have lead to an increased interest in the applicability of stem cell transplants to the repair or

replacement of damaged tissue. To improve the efficacy of such cell therapies, the tracking and homing studies of transplanted cells have been ubiquitously pursued. Magnetic resonance imaging (MRI) is a useful non-invasive test method that has high spatial and temporal resolution and has been demonstrated to be suitable for the monitoring of the in vivo behavior of cells that have been labeled with superparamagnetic iron oxide (SPIO) nanoparticles (1–3). SPIO nanoparticles have been successfully used for stem cell labeling, wherein they have been shown to not adversely impact cell physiology. Ferumoxide (Feridex[®]) is a nanoparticle that has been approved by the United States Food and Drug Administration (FDA), and it has been combined

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with transfection agents (TAs) so as to facilitate its intracellular uptake. Among the differently available TAs, protamine sulfates (Pro) have been approved by the FDA (2, 3). It has been reported that ferumoxide - Pro was more effective than ferucarbotran for cell labeling (4). It has been reported that other TAs, for example, poly-L-lysine (PLL), in combination with Ferumoxide is suitable for human stem cell labeling (5). In addition, it has been reported that when ferucarbotran (Resovist®) was used without TAs, the labeling effect was superior to that observed for Ferumoxide (3). To the best of our knowledge, the cell-labeling efficacies of ferumoxide and ferucarbotran in combination with protamine sulfate and poly-L-lysine have not been compared.

This study was performed to compare the labeling efficacies of different combinations of SPIOs and TAs and optimize a dose ratio (SPIO/TA) that facilitates the incorporation of SPIOs into the cell with no adverse effects on cell viability.

MATERIALS AND METHODS

Cell Lines and Culture Conditions

This study was approved by the institutional review board. Human adipose-derived mesenchymal stem cells (HURIM, Korea) were cultured in Dulbecco's modified Eagle's medium (DMEM) with 10% fetal bovine serum (FBS), 100 U of penicillin, and 100 μ g/ml of streptomycin (all from Invitrogen, Germany). The cells were grown in a humidified incubator with 5% CO₂ and an atmosphere at 37°C.

The cells were seeded at a density of 1×10^6 cells in a dish, and then incubated for 24 hours. The cells were labeled with the two different types of SPIOs at Fe concentrations of 25 μ g/ml or 50 μ g/ml of ferumoxide (Feridex IV; Advanced Magnetic, Cambridge, MA) and ferucarbotran (Resovist®; Schering AG, Germany). Transfection reagents (PLL; Sigma, Germany, Pro; Sigma, Germany) were added at final concentrations of 0.75 μ g/ml, 1.5 μ g/ml, 3 μ g/ml, and 4.5 μ g/ml in a serum-free culture media. The iron-labeled cells were incubated in culture media for three days.

Prussian Blue Staining

After three days of incubation, the iron-labeled cells were washed three times with PBS (phosphate-

buffered saline), air-dried on a slide glass at room temperature, and then fixed in methanol for 10 min. The fixed cells were stained in freshly prepared 5% potassium hexacyanoferrate (II) (Sigma, Germany) in 5% hydrochloric acid and then counterstained with nuclear fast red (Fluka, Kernechtrot, Germany) for 15 min.

A light microscope (Olympus, BX50, Japan) was used to determine the intracellular iron oxide distributions in the iron-labeled cells.

Cell Viability Assays

The viabilities and proliferations of the iron oxide-labeled cells (1×10^5 cells/well) were characterized by MTT (3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; Amresco, USA) assays via spectrophotometry (plate reader, Spectra Max 250, USA) at 540 nm and a trypan blue exclusion test after three days of incubation. Iron labeled adipose-derived MSCs or unlabeled control cells were seeded in 96-well plates at a density of 1×10^5 cells/well and then incubated for 24 hours. The second batch of incubated cells were treated with 10 μ l of a methylthiazol tetrazolium (MTT) dye solution and then incubated for an additional four hours. For the third batch of cells, after assaying the color of the MTT-dyed cells (purple) via microscope, 90 μ l of a detergent reagent was added to each well and the plates were kept in a dark room for two hours. For the last of the MSCs, the absorbance was measured on a plate reader at 540 nm. All of the assays were performed in triplicate. The cell viability was estimated to be 100% in comparison to the control cell group.

The Measurement of Iron Content in Iron Labeled Cells

The iron contents of the iron labeled adipose-derived MSCs were characterized via absorbance measurements by using spectrophotometry (plate reader, Spectra Max 250, USA) with a 500-nm excitation. Cell suspensions (1×10^6 cells/ml PBS) were lysed in a mixture of 35% hydrochloric acid and 65% nitric acid via heating for at least three hours at 60°C. Samples of the digested cells, along with PBS, were placed into a 96-well plate in order to calculate the iron content therein using a ferrous chloride calibration standard. In addition, a standard curve was generated for each experiment from a diluted series of

digested ferumoxide and ferucarbotran ($0.25 \mu\text{g/ml}$ and $0.5 \mu\text{g/ml}$, respectively). The average iron contents per cell were calculated as the mean value divided by the number of cells in each sample.

MR Imaging and T2 Relaxometry

To measure the sensitivity of MRI, phantom unlabeled control cells and labeled cells were harvested from each sample group (iron oxide 25 and $50 \mu\text{g/ml}$ + PLL or Pro at 0.75, 1.5, 3, and $4.5 \mu\text{g/ml}$). 3×10^5 cells obtained from a well of a 24-well plate (BD Falcon™ 24-multiwell insert systems) were suspended with 1% agarose gel and were hardened at

4°C .

The MR images were acquired with a 1.5T scanner (Signa HDX, GE healthcare, Milwaukee, WI, USA) that was equipped with an 8 channel head and neck coil. T2-weighted images were acquired using the following parameters: TR=4000; dual echo technique; TE=10/25, 40/55, 70/85, 96/110, 130/230, 330/430, 530/830, and 1130/1430; a matrix of 256×128 ; a FOV of 200 mm; 1 NEX; a 10-mm slice thickness with no gaps; and an acquisition time of 74 minutes and 40 seconds.

T2 relaxometry was obtained using MATLAB (Mathworks, Natick, MA) after defining the region of

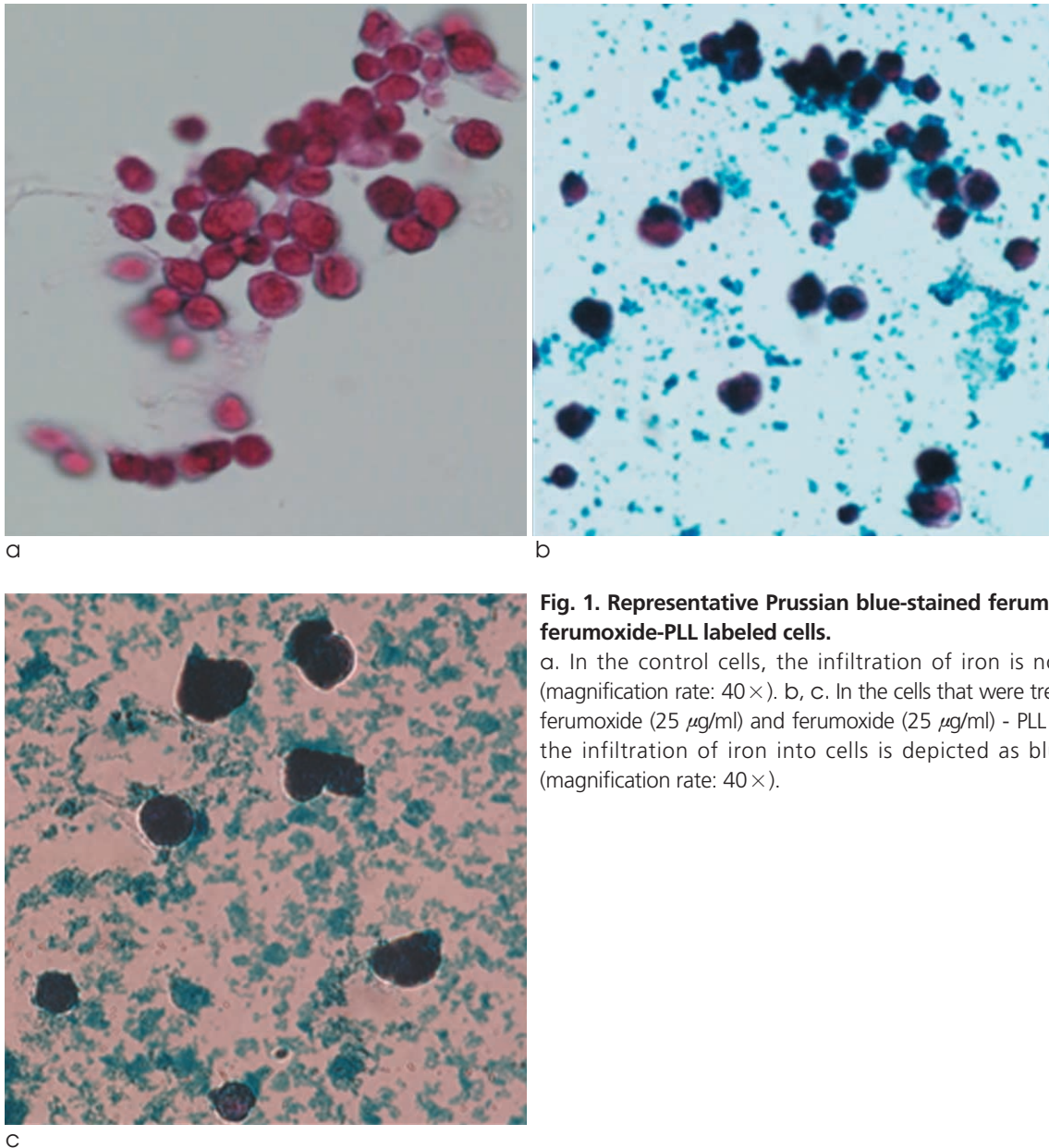


Fig. 1. Representative Prussian blue-stained ferumoxide- or ferumoxide-PLL labeled cells.

a. In the control cells, the infiltration of iron is not shown (magnification rate: $40\times$). b, c. In the cells that were treated with ferumoxide ($25 \mu\text{g/ml}$) and ferumoxide ($25 \mu\text{g/ml}$) - PLL ($3 \mu\text{g/ml}$), the infiltration of iron into cells is depicted as blue spots. (magnification rate: $40\times$).

interest (ROI) for each sample. For T2 mapping, non-linear pixel-by-pixel curve fitting was used and the following formula was used as a fitting model: $SI = M_0 e^{-\frac{TE}{T_2}}$, where SI is the signal intensity and M_0 is the initial amplitude of the net magnetization. T_2 is the transverse relaxation time and TE is the echo time. Among these parameters, M_0 and T_2 are calculated via non-linear pixel-by-pixel curve fitting using 16 variable TE_s and 16 variable SI_s , which are dependent in each TE .

Statistical Analysis

The iron contents and cell viabilities of the samples were characterized in triplicate, whereas the T2 relaxation times were evaluated in duplicate. The investigated experimental groups (iron contents, viabilities, T2-relaxation times) were classified into four groups according to various treatments of MR contrast agents and TAs. A statistically significant difference was found with one-way ANOVA, followed by an Fisher's Least Significant Difference (LSD) test at a value of $p < 0.05$.

RESULTS

Cell Labeling and Cytological Analysis

To assess cell labeling via Prussian blue staining, micrographs of the labeled cell groups were compared to unlabeled control cells. From these micrographs, it was observed that iron particles had infiltrated the

cytoplasm of the labeled cell group. In the unlabeled control cells, the iron particle infiltration phenomenon could not be observed via Prussian blue staining (Fig. 1).

Cellular Viability

Regardless of TA concentration, the cell viabilities in the SPIOs groups (ferumoxide or ferucarbotran) that were labelled with TAs were significantly decreased in comparison to that of the unlabelled group ($p < 0.01$) (Fig. 2). In cells that were labelled with 25 $\mu\text{g/ml}$ of ferumoxide and PLL and 50 $\mu\text{g/ml}$ of ferumoxide and Pro, the viability was relatively high. Viability, as a function of TA concentration, did not appreciably differ ($p > 0.05$). Nevertheless, in cells labelled with ferucarbotran, the cell viabilities were observed to have substantially decreased ($p < 0.01$) (Table 1).

The Measurement of Iron Contents in Labeled Cells

The iron contents of cells that had been treated with SPIOs and TAs were observed to have significantly increased in comparison to cells that were only treated with SPIOs ($p < 0.01$) (Fig. 3), regardless of TA concentration. The group that was treated with ferumoxide exhibited higher overall iron contents in comparison to the group that was treated with ferucarbotran ($p < 0.01$). Particularly, cells that were treated with ferumoxide at a concentration of 50 $\mu\text{g/ml}$, regardless of TA type or concentration, maintained high iron contents. Cells that had been treated with ferucarbotran demonstrated no significant differences

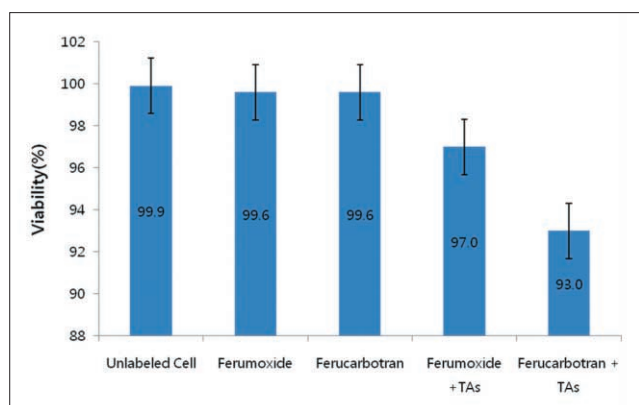


Fig. 2. Graphs of cellular viability, as measured by MTT assay.

There was no significant difference between ferumoxide-labeled cells and ferucarbotran-labeled cells; however, cell viability was lower with ferucarbotran-TA in comparison to ferumoxide-TA.

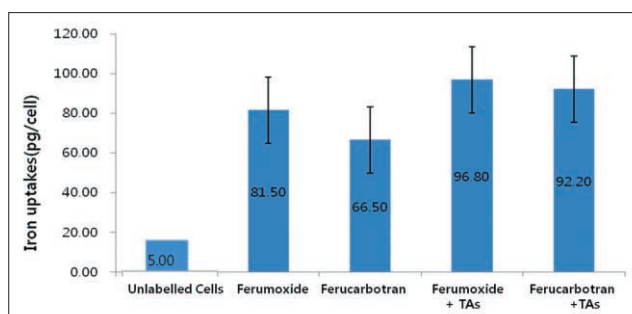


Fig. 3. Quantitative analysis of cellular iron content.

In the groups that were treated with TAs, the iron contents were observed to have significantly increased in both groups that were labeled with ferumoxide or ferucarbotran ($p < 0.01$). The group that was labeled with ferumoxide exhibited an overall higher iron content in comparison to the group that was labeled with ferucarbotran ($p < 0.01$).

in iron content as a function of SPIO concentration or TA type ($p > 0.05$); however, iron content was observed to increase as a function of increasing TA concentration (Table 1).

MR Imaging and T2 relaxation time

Cells that were labeled with SPIOs exhibited significantly shorter T2 relaxation times in comparison to those of unlabeled cells ($p < 0.01$). Furthermore, cells that had been labelled with TAs exhibited significantly

shorter T2 relaxation times in comparison to untreated cells ($p < 0.05$) (Fig. 4A). Shorter T2 relaxation times were observed for ferumoxide supplementation than for ferucarbotran supplementation ($p < 0.05$).

Regardless of ferumoxide concentration, in the ferumoxide-labeled group, the cells that were treated with PLL exhibited significantly shorter T2 relaxation times in comparison to cells that were treated with Pro ($p < 0.01$). Cells that were treated with 25 $\mu\text{g/ml}$

Table 1. Cell Viabilities, Iron Contents, and T2 Relaxation Times According to the Combination of SPIOs and TAs

Agents + TAs (PLL/Pro) Concentrations ($\mu\text{g/ml}$)	Viability (%)	Iron contents (pg/cell)	T2 relaxation time (msec)
	PLL / Pro	PLL / Pro	PLL / Pro
Fe 25 + PLL / Pro 0.75	98.4 / 98.6	80.9 / 96.6	40.2 / 56.3
PLL / Pro 1.5	99.3 / 97.6	96.5 / 92.4	10.7 / 62.5
PLL / Pro 3	99.7 / 97.3	106.9 / 96.5	11.9 / 54.5
PLL / Pro 4.5	93.1 / 91.9	97.2 / 92.4	15.2 / 52.6
Fe 50 + PLL / Pro 0.75	100.0 / 95.6	86.2 / 91.1	76.9 / 53.6
PLL / Pro 1.5	98.1 / 98.7	109.2 / 94.0	26.1 / 50.0
PLL / Pro 3	92.2 / 99.2	110.7 / 106.2	12.5 / 42.9
PLL / Pro 4.5	96.1 / 98.3	98.5 / 93.6	8.40 / 41.9
Re 25 + PLL / Pro 0.75	92.6 / 95.9	78.1 / 91.6	76.8 / 54.5
PLL / Pro 1.5	94.3 / 93.8	89.1 / 93.6	21.4 / 15.2
PLL / Pro 3	94.8 / 94.6	96.6 / 97.0	36.9 / 4.5
PLL / Pro 4.5	97.7 / 95.7	94.5 / 94.5	27.7 / 5.4
Re 50 + PLL / Pro 0.75	93.7 / 91.3	74.5 / 90.9	35.7 / 68.7
PLL / Pro 1.5	92.7 / 92.4	95.1 / 94.5	14.3 / 43.8
PLL / Pro 3	90.4 / 91.5	92.9 / 107.4	20.5 / 44.9
PLL / Pro 4.5	93.0 / 91.3	96.4 / 92.7	19.6 / 16.9

Note.— SPIOs: superparamagnetic iron oxide, Fe: ferumoxide, Re: ferucarbotran, PLL: poly-L-lysine, Pro: protamine sulfate.

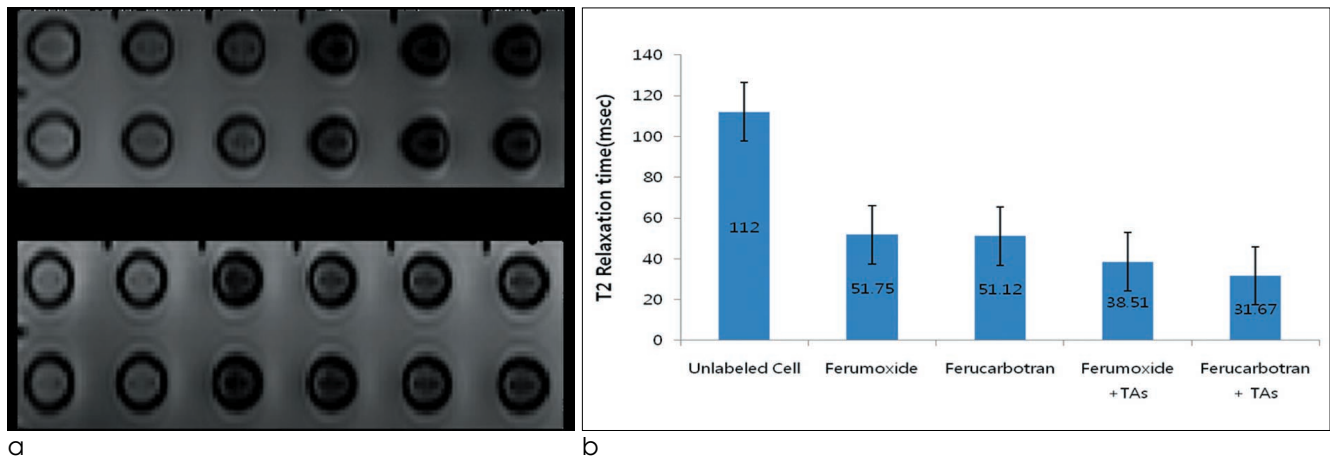


Fig. 4. T2-weighted MR images of the phantoms and T2 relaxation time graph.

a. Ferumoxide 25 $\mu\text{g/ml}$ - TA depicted a gradual decrease of signal intensity as a function of TA concentration on T2 weighted MR image. Ferucarbotran 25 $\mu\text{g/ml}$ - TA depicted a subtle decrease of signal intensity. b. The group that was treated with TAs exhibited significantly shorter T2 relaxation times in comparison to the untreated group ($p < 0.05$).

ferumoxide with 3.0 $\mu\text{g/ml}$ PLL or Pro exhibited the short T2 relaxation times. In ferucarbotran-treated cells, regardless TA concentration, the cells that had been treated with 25 $\mu\text{g/ml}$ ferucarbotran exhibited shorter T2 relaxation times in comparison to cells that did not receive TA supplementation. The shortest T2 relaxation time was observed at a ferucarbotran concentration of 25 $\mu\text{g/ml}$ and a Pro concentration of 3 $\mu\text{g/ml}$ (Table 1, Fig. 4b).

DISCUSSION

Magnetic resonance imaging (MRI) is a useful non-invasive test method that has high spatial and temporal resolution and has been demonstrated to be suitable for the monitoring of the in vivo behavior of cells that have been labeled with superparamagnetic iron oxide (SPIO) nanoparticles (1-3). But, the limited resolution of MRI requires the presence of a sufficient cell numbers to be imaged, resulting in improper investigation about the individual cell behavior and cell-cell interaction.

The incorporation of SPIO nanoparticles into cells and the monitoring of the migration of magnetically labeled cells via cellular MRI have been investigated (6-9). Iron oxide-labeled cells appear as hypointense areas in tissues with an associated susceptibility artifact or amplification of the decreased signal intensity on iron-sensitive T2-weighted and T2*-weighted gradient echo images (1, 7, 9-13). Relaxation rate ($R2^*$) to iron oxide-loaded cells has been reported to be 70 times greater than that for Relaxation rate ($R2$) (14).

To achieve the intracellular uptake of commercially available SPIO nanoparticles, TAs have been needed in most studies (1, 6, 15, 16). The FDA-approved agent ferumoxide (Feridex[®]) is a suspension of dextran-coated SPIO that has been used as an MRI contrast agent and has a negative zeta potential due to the presence of dextran carboxyl groups (11). When ferumoxide is complexed to TA so as to modify the zeta potential of the SPIOs, efficient and effective cell labeling can be obtained, which allows for the detection of labeled cells via MRI (10, 11, 17).

In ferucarbotran (Resovist[®]), the magnetic nanoparticle aggregates therein are stabilized by carboxydextran, whereas in ferumoxide, this is achieved via non-

functionalized dextran. Ferucarbotran has been reported to be spontaneously taken-up without the need for a TA (3). TAs like PLL and Pro are mostly cationic and positively charged molecules (3). Pro is a commercially available, FDA-approved agent and is typically used to reverse heparin anticoagulation (1). Pro is well-tolerated by cells, with a high therapeutic window of more than 50 mg/ml (1), and does not alter the viability and functional capacity of a variety of cell types (18). Sorigi et al. have shown that when Pro is complexed to DNA, it is approximately 100 times more efficient than PLL in cell transfection (19). PLL has a relatively narrow tolerated concentration of 10 $\mu\text{g/ml}$ or less in media before causing significant cell death (1, 6) and furthermore, it inhibits the chondrogenic differentiation capacity of mesenchymal stem cells (20). The combination of excessive amounts of polycations with cells results in pore formation in the cell membrane and an intracellular ionic imbalance that can ultimately lead to cell death (21, 22). Ferumoxide-PLL has been reported to potentially increase the formation of reactive oxygen species and hydroxyl-free radicals and may increase the rate of apoptosis or cell death (6, 9, 17). Additionally, residual ferumoxide-PLL may remain on the surface of cells or clump cells together in the final cell preparation prior to infusion (6); however, ferumoxide-PLL has been shown to effectively label cells, and several studies have reported on the safety profile of this complex, which has exhibited no difference in cell viability, growth rate, and apoptotic index in comparison to unlabeled cells (6, 17, 22). Ferumoxide concentrations of 25 to 50 $\mu\text{g/ml}$, at a ratio of ferumoxide to PLL that ranged from 1:0.03 to 1:0.05 $\mu\text{g/ml}$, have been demonstrated to be efficient in the labeling of MRI-detectable cells (6, 17). In the present study, cells that were treated with ferumoxide at a concentration of 25 $\mu\text{g/ml}$ exhibited higher cellular viabilities than those treated with ferumoxide at 50 $\mu\text{g/ml}$. When TAs were added, the cells that were labeled with ferumoxide exhibited higher cellular viabilities than those that were labeled with ferucarbotran ($p < 0.05$). Cellular iron concentration was higher when 1.5 $\mu\text{g/ml}$ and 3 $\mu\text{g/ml}$ concentrations of transfection agent were used. An effective labeling was obtained via 25 $\mu\text{g/ml}$ of ferumoxide and 3.0 $\mu\text{g/ml}$ of TA in the present study, which is consistent with the results of previous studies (6, 17). The magnetic labeling of cells with ferumox-

ide-protamine sulfate complexes is comparable or superior to other ferumoxide-TA combinations (1, 6, 11). In the present study, there were also no significance differences in the cellular viability and intracellular iron content between PLL and Pro supplementation.

The average iron content of the SPIOs-TA-treated cells was 94 ± 1.93 pg/cell, which was higher than that of SPIOs-treated cells. The observed iron amount in the present study was higher than that observed in a previous study(1); however, ferumoxide-Pro ratios ranging from 50:1.5 μ g/ml to 50:6.0 μ g/ml resulted in cellular iron incorporation that was similar to that achieved via ferumoxide-Pr (1) and ferumoxide-PLL labeling (1, 11, 17).

In regard to mesenchymal stem cell labeling, high concentrations of ferucarbotran demonstrated efficient labeling without TAs. This work demonstrates that when using ferucarbotran (25 μ g/ml), there are some intracellular deposits of iron (iron amount < 5 pg/cell), whereas with ferumoxide (25 μ g/ml), there are little or no nanoparticle deposits (iron amount < 1 pg/cell). When using ferucarbotran (25 μ g/ml) and PLL (1.5 μ g/ml), the iron concentration was approximately 15 pg/cell.

Measured iron concentrations ranged from 20 to 25 pg/cell in cells that were treated with ferumoxide (25 μ g/ml)-PLL (1.5 μ g/ml) complexes, which was higher than those achieved using ferucarbotran (25 μ g/ml)-PLL (1.5 μ g/ml) complexes. The iron content per cell using ferucarbotran (25 μ g/ml) was higher than that using ferucarbotran (25 μ g/ml) – PLL (1.5 μ g/ml) (3). In the present study, there was no significant difference in cellular viability between the ferumoxide- and ferucarbotran-treated cells; however, ferucarbotran-TA-treated cells exhibited significantly lower cellular viabilities in comparison to those of ferumoxide-TA-treated cells. The iron content was higher in ferumoxide- in comparison to ferucarbotran-treated cells (81.5 ± 14.42 pg/cells vs. 66.5 ± 2.125 pg/cells, respectively), which was higher when compared with a previous study (3). Cellular iron concentrations did not significantly differ between the two investigated SPIO-TA complexes in the present study.

T2 and T2* relaxation times have been measured in some studies in order to evaluate cell labeling efficiency (2, 11, 17, 23, 24); however, cell densities or incubation time-related changes have been the

primary focuses of these studies. Janic et al. analyzed the R2* values (1/sec) that were created from T2* images of ferumoxide - Pro-labeled cells and observed concentration-dependent T2* relaxation time changes. Furthermore, they identified that 100 μ g/ml of Pro in combination with 3 μ g/ml of ferumoxide was efficient to label cells for cellular MRI (2). In the present study, the T2 relaxation time did not significantly differ as a function of SPIO and TA type and SPIO concentration. The T2 relaxation time did reveal significant changes among the different TA concentrations. T2 relaxation times significantly decreased as a function of increasing TA concentration, reaching a low value at a TA concentration of 3.0 μ g/ml.

There are some limitations to our study. First, in vitro data on cell samples in agar phantoms that were imaged at 1.5T did not provide the same result for an in vivo situation. Second, cellular viability might depend on the incubation period, and this study was performed after only three days of incubation.

In conclusion, cells that are treated with ferumoxide - TA demonstrate higher cellular viabilities and higher iron contents in comparison to cells treated with ferucarbotran - TA. Ferumoxide at a concentration of 25 μ g/ml in combination with PLL or Pro at a concentration of 3.0 μ g/ml would be sufficient to label mesenchymal stem cells.

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인체 중간엽 줄기세포의 표지를 위한 상용화 된 Superparamagnetic Iron Oxide Nanoparticle과 Tansfection Agent의 적절한 병용을 위한 연구

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목적: 상용화 된 superparamagnetic iron oxide (SPIO) nanoparticles과 transfection agent (TA)의 최적의 병용 용량을 알아보고자 하였다.

대상과 방법: Protamine sulfate (Pro), poly-L-lysine (PLL)과 ferumoxide, ferucarbotran을 다양한 농도에 서 인체 중간엽 줄기세포에서 배양하여 세포 생존능을 알아보았다. 세포 철 섭취율은 정성적으로, 정량적으로 분석하였다.

결과: Ferumoxide 처리군의 생존능과 철 섭취율은 ferucarbotrn 처리군보다 통계적으로 의미있게 높았다 ($p < 0.05$). T2 이완시간은 ferumoxide 처리군에서 짧았다 ($p < 0.05$). 25 $\mu\text{g}/\text{ml}$ ferumoxide와 3.0 $\mu\text{g}/\text{ml}$ Pro 또는 PLL 병용군이 최적의 조건이었다.

결론: Ferumoxide 처리군의 세포 생존능과 철 섭취율은 ferucarbotrn 처리군보다 높았다. 25 $\mu\text{g}/\text{ml}$ ferumoxide와 3.0 $\mu\text{g}/\text{ml}$ TA는 줄기세포 표지에 적합하다.

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