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Original Article

A comparative study of transfection of rat mesenchymal stem cells using polyethyleneimine-coated magnetic ferro-ferric oxide nanoparticles and lipofectamine

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Abstract: Stem cell transfection agents have traditionally included lipofectamine, but newer agents include polyethyleneimine-coated magnetic ferro-ferric oxide nanoparticles. This study was done to compare these two methods in transfecting cultured ratmesenchymal stem cells. Magnetic ferro-ferric oxide nanoparticles were coated with polyethyleneimine using co-precipitation of alkali metal ions, forming a mobility diameter of approximately 50 nm. It dispersed well in water. Compared to the lipofectamine, polyethyleneimine-coated magnetic ferro-ferric oxide nanoparticles had higher transfection rate-in rat mesenchymal stem cells ($P<0.05$). Meanwhile, cell proliferation analysis revealed less toxicity of such nanoparticles to rat mesenchymal stem cells. In summary, polyethyleneimine-coated magnetic ferro-ferric oxide nanoparticles are safe and had a higher transfection rate in cultured ratmesenchymal stem cells, indicating that this improved technique of mesenchymal stem cell transfection may have clinical application potential.

Keywords: Mesenchymal stem cells, stem cells, transfection, magnetic nanoparticles, lipofectamine, gene therapy

Introduction

The human mesenchymal stem cells (hMSC) have the potential to differentiate into a variety of non-epithelial differentiated cells, including osteoblasts, vascular endothelial cells, and chondrocytes [1]. For this reason, hMSC have an important role in human gene therapy [2]. With the increasing use of hMSC in gene therapy, some recent studies have been done to develop safe and efficient transfection vectors [3].

Traditional vectors in gene therapy fall into two categories: viral vectors and non-viral vectors [4, 5]. Commonly used viral vectors include retroviral vectors, adenoviral vectors, and adeno-virus-associated vectors [6]. Until recently, viral vectors have been the most efficient transfection vectors in gene therapy [4], with a transfection rate reported to be as high as 90% in some studies.

In up to 70% of gene therapies used in clinical trials, viral transfection vectors have been used [7]. However, virus vectors also have their drawbacks, including difficulty in production, insertion of mutations into the genome, and in some cases triggering severe immunological reactions [8, 9]. Non-viral transfection vectors are less likely to trigger immune reactions, and they have low toxicity. Because of these advantages, an increasing number of studies have been done to develop and evaluate non-viral transfection vectors for gene therapy [5].

Although a variety of non-viral transfection vectors have been developed and studied in the past ten years, most of these vectors have shown a low transfection rate [10]. Recently, nanoparticles have been adopted in the development of non-viral transfection vectors [11, 12]. Due to their unique chemical and physical properties, nanoparticles can combine with and condense DNA and RNA, and import DNA

or RNA into many types of cells [13]. Because nanoparticles are non-biological and non-immunogenic, they do not trigger immune reactions. Importantly, nanoparticles have higher transfection rate [14]. In particular, magnetic nanoparticles have shown wide applications in biomedical research. These applications include the detection of small molecules, therapeutic drug targeting to cells, and cell imaging [15-19]. Magnetic nanoparticles can covalently bind with DNA, and under the effects of a magnetic field, they have shown higher transfection rates [20-26].

The purpose of this study was to evaluate and compare the transfection of cultured rat mesenchymal stem cells (MSC) using polyethyleneimine (PEI)-coated magnetic ferro-ferric oxide nanoparticles (Fe_3O_4 NPs) and lipofectamine.

Materials and methods

Synthesis of polyethyleneimine (PEI)-coated magnetic ferro-ferric oxide nanoparticles (Fe_3O_4 NPs)

The following formula was used for synthesizing polyethyleneimine-coated magnetic ferro-ferric oxide nanoparticles (PEI-coated Fe_3O_4 NPs):

$$2Fe^{3+}+Fe^{2+}+8OH^{-}\rightarrow Fe_3O_4+H_2O$$

An alkaline co-precipitation method included a total of 1.352 g $FeCl_2$ and 0.497 g $FeCl_3$, dissolved in 30 ml of de-ionized water, and dispersed using ultrasound for 3 minutes. The mixture was filtered into a 100 ml three-neck flask using a 0.22 μm filter. Nitrogen was bubbled through the liquid for 10 minutes. After being cooled down to 2-4°C using ice water, the liquid was mixed at a speed of 500 rpm in the three-neck flask while 10 ml of precipitation liquor was added quickly.

The precipitation liquor was made by adding 2.5 ml of ammonia and 0.5 g 25 KD of dendroid polyethyleneimine (PEI)-25Br (Sigma-Aldrich, US) to 10 ml of de-ionized water. After the precipitation liquor was added, the mixture was heated to 80°C over 15 minutes and stirred continuously for 2 hours. After the reaction was over, the mixture was cooled down to 25°C for 15 minutes, stirred continuously for 1 hour, and dispersed under 20 KHZ, 75 mW ultrasound for 10 minutes. The mixture was dialyzed using de-ionized water for 2 days, and any uncombined PEI was removed. Finally, the mixture was sterilized with 25 KGy (^{60}Co) and stored at 4°C.

Determination of Fe using 1,10-phenanthroline monohydrate

The PEI-coated Fe_3O_4 magnetic nanoparticles were viewed using a transmission electron microscope and the microstructure and size of the particles were studied. The ξ-potentials and diameters of the particles were measured with dynamic light scattering (DLS). Highly diluted samples were put into a cuvette, which was placed into a laser particle size analyzer to determine ξ-potentials and particle diameters.

Culture and identification of rat bone marrow mesenchymal stem cells (MSCs)

Bone marrow mesenchymal stem cells (MSCs) were separated and purified as previously described [27]. Briefly, a one-month-old Wistar rat (provided by the animal experiment center, Jilin University) was sacrificed, and the femur and tibia were removed immediately after death. The distal epiphyses were removed, and the bone marrow fluid was rinsed into a centrifuge tube with serum-free Dulbecco's modified Eagle's medium (DMEM) (Gibco, Ltd.). All the animal experiments were carried out in compliance with the guidelines and practices established by the Institutional Animal Care and Use Committee of Jilin University.

Mesenchymal cells were obtained by centrifuging the bone marrow fluid with 1.08×10^3 g/L percol-layered liquid, 10% fetal bovine serum (FBS), DMEM, 100 U/mL of penicillin and 100 U/mL of streptomycin. The cell density was adjusted to 1×10^6/ml, and the cell suspension was transferred to a culture bottle, stored at 37°C in an incubator with 5% CO_2 and saturated humidity. The DMEM was changed after two days, and then on every three days. After the cells had become 80% confluent, they were digested with 0.25% pancreatin and 1 mM EDTA. The cells were sub-cultured with a ratio of 1:2.

Rat mesenchymal stem cells (MSC) transfection using pACCMV-EGFP plasmids

The pACCMV-EGFP plasmid was provided by Prof. Changyu Zheng from the National Institute

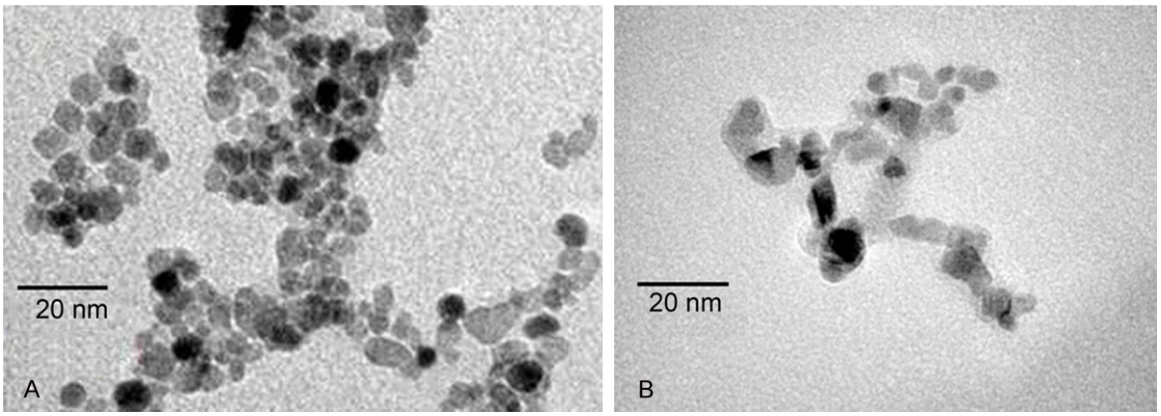

Figure 1. Transmissionelectronmicrograph. A. Fe_3O_4 nanoparticles. B. Polyethyleneimine (PEI)-coated Fe_3O_4 nanoparticles.

Table 1. Diameter of particles and ξ-potentials

	Size (nm)	ξ-potential (mV)
Fe_3O_4 nanoparticles	29.0±6	-26.7±1.6
PEI modified Fe_3O_4 nanoparticles	79.6±25	46±0.5
PEI^+-DNA	310.7±4	14.4±3
DNA magnetic complex (EGFP)	218.2±55	28.3±2

Polyethyleneimine (PEI); enhanced green fluorescent protein (EGFP).

for Dental and Craniofacial Research (NIDCR), Bethesda, MD, US. The cultured MSCs were digested by 0.25% pancreatin, washed twice with PBS and re-suspended in DMEM medium. The number of cells was counted, the MSC concentration was adjusted to 1×10^4 per well, and the cells were transferred to a 12-well culture plate. The serum-free medium was changed the following day. Then, 2.4 μg of plasmid DNA was placed in a sterile tube and dissolved in 100 μl of serum-free medium.

The ratio of PEI-coated Fe_3O_4 nanoparticles to plasmid DNA was 0.25, 0.5, 0.75, 1.0 and 1.25 (μg/μg), respectively. This mixture was stored at room temperature, mixed and then combined with the plasmid. A combined compound of DNA-magnetic nanoparticles was formed following incubation at room temperature for additional 20 minutes. This compound was added into DMEM medium, under which aNd-Fe-B magnet was placed and stored in an incubator at 37°C and 5% CO_2. After 5 hours, the medium was changed to 10% FBS in 1640 medium. Each combination was transfected in 5 culture wells; MSCs transfected by Lipofectamine™ 2000 (Invitrogen, Grand Island, NY, USA) were used as controls.

Enhanced green fluorescent protein (EGFP) was observed using a fluorescence microscope following transfection. The efficacy of transfection was measured using flow cytometry following pancreatin digestion.

MSC cell growth measured by the MTT Cell Proliferation Assay

The MTT Cell Proliferation Assay was performed using kit from Fluka, Ltd. The third generation of rat MSCs was transferred to a 96-well culture plate with a cell density of 5×10^3. There were three experimental groups: the first group was the MSCs transfected by PEI-coated Fe_3O_4 magnetic nanoparticles, the second group was the MSCs transfected by lipofectamine, and the third group was the non-transfected MSC cells as control. MTT tests were conducted on the 1st, 2nd, 4th, 6th, and 8th day, and cell growth curves were produced.

Statistical analysis

The statistical software package SPSS 13.0 was used to analyze the data. Continuous variables were analyzed using the mean and standard deviation. Chi-square tests were used to compare the transfection rates. A statistical result of $P<0.05$ was considered significant.

Results

Polyethyleneimine-coated magnetic ferro-ferric oxide nanoparticles (PEI-coated Fe_3O_4 NPs)

Transmission electron micrographic images of the synthesized Fe_3O_4 nanoparticles are shown

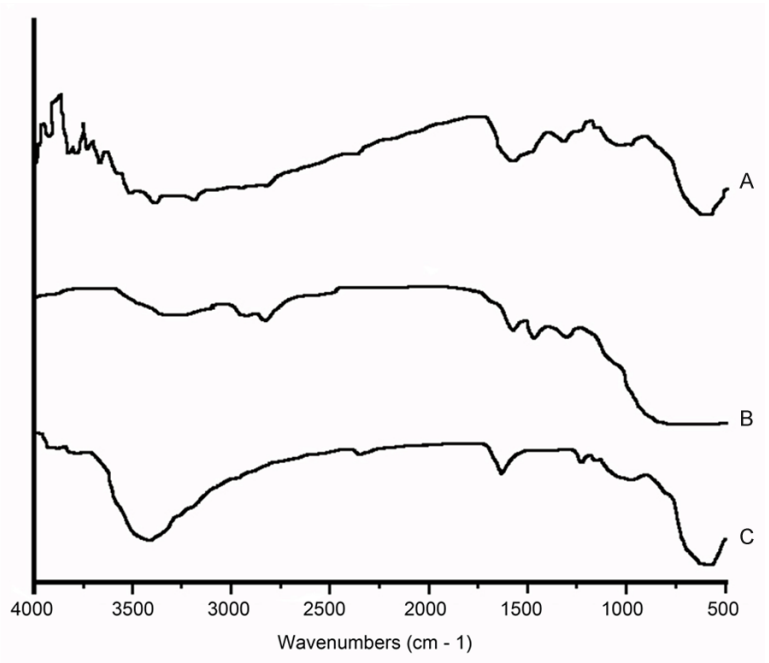

Figure 2. Infrared spectrograms for Fe_3O_4, polyethyleneimine (PEI) and PEI-coated Fe_3O_4 nanoparticles. A. PEI-coated Fe_3O_4 nanoparticles. B. PEI. C. Fe_3O_4 particles.

in **Figure 1**. The Fe_3O_4 nanoparticles are shown in **Figure 1A**, and the PEI-coated Fe_3O_4 nanoparticles are shown in **Figure 1B**. The Fe_3O_4 nanoparticles are round and have a diameter of 10 nm. There is a polymeric shell around the PEI-coated Fe_3O_4 nanoparticles.

When compared to Fe_3O_4 nanoparticles, PEI-coated Fe_3O_4 magnetic nanoparticles were also more dispersive in water. Plasmid DNAs are considered to be anionic polyelectrolytes, with a large amount of negative surface charge due to the presence of phosphate residues.

The size of the Fe_3O_4 nanoparticles and the ξ-potentials are shown in **Table 1.** At pH 7.0, the potentials were -26.7±1.6 mV because there were large amounts of OH^- ions present. But potentials were +46±0.5 mV for PEI-coated Fe_3O_4 nanoparticles due to the presence of NH_4^+ions supplied by PEI. Potentials for the combination of Fe_3O_4-PEI-Plasmid (FPP) were +28.3±2 mV, which was significantly different from the potentials for the PEI-coated Fe_3O_4 nanoparticles (P<0.05). Positive charges were neutralized after combining with the plasmids. The remaining positive charges contribute to the combination of FPP and cell membrane. Previous studies have shown that, compared to molecules with negative charges, molecules with positive charges are more likely to combine with cell surfaces and, therefore, to be taken up by the cells more easily [28].

The infrared spectrograms for all particles are shown in **Figure 2**. The spectrum for Fe_3O_4-measured by a Fourier transformation infrared spectrometer (FT-IR) (**Figure 2A**), indicating an absorption peak of Fe_3O_4 under 576 cm^{-1}. The infrared spectrogram for PEI (**Figure 2B**) shows absorption peaks under 2830 cm^{-1} and 2940 cm^{-1} due to the presence of C-H bonds. The PEI-coated Fe_3O_4 nanoparticles (**Figure 2C**) show absorption peaks characteristic for both Fe_3O_4 and PEI: 576 cm^{-1} (Fe_3O_4), 3470 cm^{-1} (-OH), 2830 and 2940 cm^{-1} (-CH_2-). Comparison of these spectrograms confirms that PEI-coated Fe_3O_4 nanoparticles have been produced.

Culture and amplification of rat bone marrow MSCs

Most of primary culture cells became attached to the culture wells within 24 hours after inoculation. After 2-3 days, the MSCs were spindle-shaped, polygonal and formed colonies (**Figure 3A**). The growth and confluency of the MSCs that occurred in 8 to 10 days were observed (**Figure 3B**). After subculturing, the shape of the MSCs became more uniform.

MSCs transfected by PEI-coated Fe_3O_4 magnetic nanoparticles

After transfection, fluorescence microscopy showed that the ratio of PEI-coated Fe_3O_4 nanoparticles to plasmid was 0.75, representing a high transfection efficacy. After 24 hours, the intensity of enhanced green fluorescent protein (EGFP) was higher in the PEI-coated Fe_3O_4 nanoparticles than in lipofectamine-transfected group (**Figure 4A** and **4B**). The transfection efficacy was 4.7% and 12.9% in the lipofectamine-transfected group and PEI-coated Fe_3O_4 nanoparticles, respectively, as

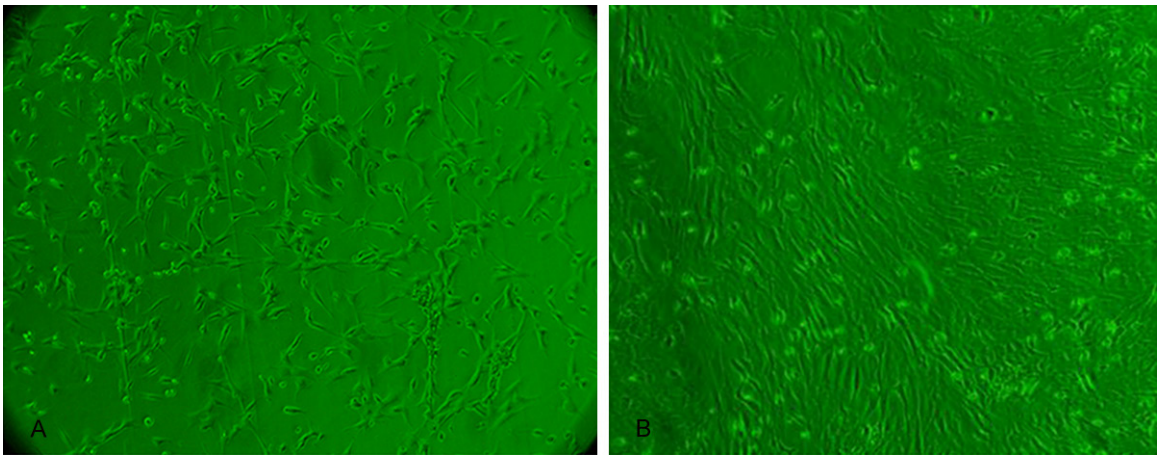

Figure 3. Phase contrast micrographs showing the morphology of the cultured rat mesenchymal stem cells (MSCs). A. After 3 days of culturing, the MSC become spindle-shaped, polygonal, and form colonies. B. MSC after reaching the confluence (8 to 10 days).

shown by flow cytometry. The efficacy of transfection in the treatment group was statistically different from that in the control group (**Figure 4C** and **4D**).

The prerequisite for nanoparticles to function as a gene carrier is their ability to coat the cell surface with a positive charge. This means that they can be combined with negatively charged DNA. The major groups providing positive charges include amino (-NH_2), imino (-NH-) and = N-. At physiological pH, nanoparticles are more likely to be coated with positive charges.

Cell viability measured by MTT test

Compared to control group, the treatment group had smaller effect on cell growth; lipofectamine had a great effect on cell growth (**Table 2**).

Discussion

Gene therapy can be defined as the introduction of target genes into cells *in vivo* for the purpose of altering the course of a medical condition or disease. Human mesenchymal stem cells (hMSCs) have been shown to have a role in the treatment of diseases. For example, hMSCs derived from autologous bone marrow have been successfully used in repairing maxillofacial injuries [29, 30]. Naked DNA is less likely to enter into cells and more likely to be degraded by nucleases. Therefore, it is critical to have carriers that can introduce target genes into cells and that results in sustained effects.

Nanoparticles measure between 1 to 100 nanometers in size. Recent research has demonstrated a wide variety of potential applications for nanoparticles in medicine [31-33]. Nanomaterials can combine and concentrate DNAs and RNAs and then introduce them into cells [13]. In addition, nanomaterials do not induce an immune response, and transfection efficacy is higher because of their special physicochemical properties.

The polyethyleneimine (PEI) coating contains a polymer, which by the method used in this study would be expected to produce magnetic particles of a smaller size that can be absorbed more easily by the stem cells [34, 35]. Particles less than 200 nm in diameter can enter the cells via pinocytosis. Nanoparticles that coat the cell membrane facilitate DNA entry into cells, which will be degraded. This process can be delayed by a stereo-electronic effect and electrostatic repulsion and by a 'proton sponge' effect of the surface polymer characterized by polyethyleneimine [13, 36]. Proton pump acidity or lysosome fusion during endocytosis can reduce the pH and facilitate DNA degradation. Carriers coated with secondary/tertiary amine may buffer the pH in lysosomes, delay DNA degradation and increase the possibility of entry into the cytoplasm. Also, because of a sustained buffering effect, lysosomes will become swollen, lysosome membranes will rupture, and the nanoparticles will be released.

Currently, the underlying mechanisms of how DNA dissociates from nanoparticles are not well understood. Some studies report that DNA may be released during the process of plasmid buffering; other studies support the view that

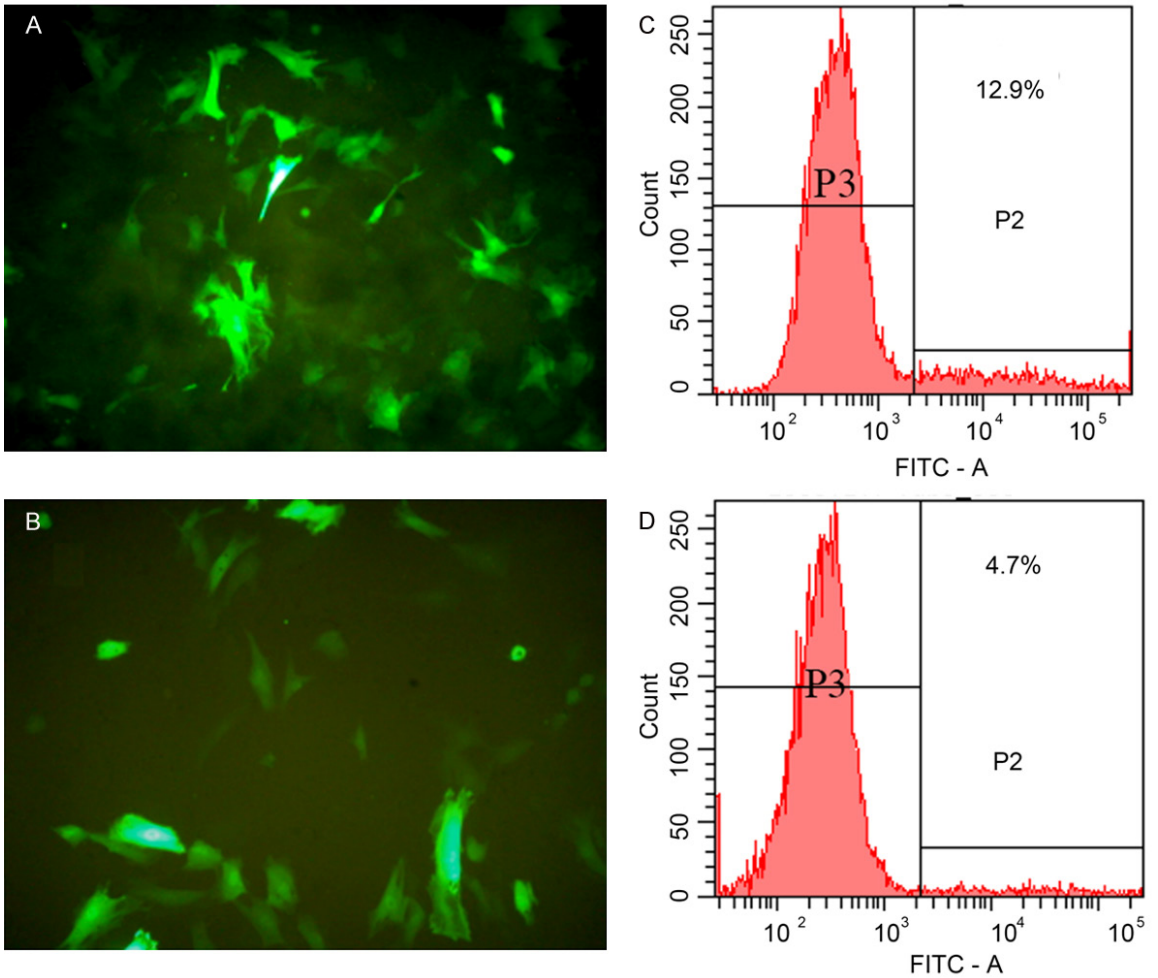

Figure 4. Transfection efficiency. A. Transfected by magnetic Fe_3O_4 (shown using fluorescence inverse microscopy). B. Transfected by liposomes (shown using fluorescence inverse microscopy). C. Transfection efficacy was 12.9% for magnetic PEI-coated Fe_3O_4 nanoparticle-transfected group, as measured by flow cytometry. D. Transfection efficacy was 4.7% for lipofectamine-transfected group, as measured by flow cytometry.

Table 2. MTT test determined the relative cell viability

	pACCMV-EGFP (%)
PBS	100±2.5
Lipofectin	85.5±1.9
PEI modified Fe_3O_4 nanoparticles	90.4±2.3*

Polyethyleneimine (PEI). *Indicates significance (P<0.05) compared with Lipofectin groups.

DNA may be released during the transfer process of nanoparticles from the cytoplasm to the nucleus. In this study, the use of infrared spectrograms confirmed the successful synthesis of PEI-coated Fe_3O_4 nanoparticles.

Here, as compared to lipofectamine-mediated transfection, polyethyleneimine-coated magnetic ferro-ferric oxide nanoparticles showed a higher transfection rate in cultured rat mesenchymal stem cells.

The data presented in this study suggest that as compared to transfection using lipofectamine, polyethyleneimine-coated magnetic ferro-ferric oxide nanoparticles had a higher transfection rate in cultured human mesenchymal stem cells. This improved technique of mesenchymal stem cell transfection may have future applications in human stem cell therapy.

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Disclosure of conflict of interest

None.

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