Targeted Delivery of Polyclustered-SPION Labeled Mesenchymal Stem Cells in Ototoxic Hearing Loss Mouse Model

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Abstract

Background

Highly specialized cells with self-renewal and differentiation potentials are known to as stem cells. These cells can keep their stemness or develop into more specialized cells. Mesenchymal stem cell (MSC)-based cell treatment has caught researchers' interest due to its lack of ethical problems, simplicity of isolation, and abundance. The use of stem cell treatment in regenerative medicine has recently been a subject of great interest. In vitro and in vivo, MSCs have been shown to have anti-inflammatory and immunomodulatory properties. The ability of MSCs to decrease T-cell proliferation and reduce T-cell functioning is responsible for their potent immunosuppressive effects. They possess potential to home to injury site and stimulate indigenous cells to repair and rebuild it, despite their homing ability delivering the stem cell to the anatomically complex area such as inner is still remains as challenge. Studies on enhancing the delivery of stem cells has been emerging.

Method

The Superparamagnetic iron oxide nanoparticles (SPION) with an iron oxide-based nanoparticle cluster core covered with PLGA-Cy5.5 has been developed. To allow PCS nanoparticles to be transported into MSCs by endocytosis, we modified the nanoparticles for internalization. Nanoparticle labeled mesenchymal stem cells are administered in ototoxic mouse model with or without magnetic field.

Results

As an outcome, when compared to the control groups, the intratympanic administration with magnet group had the most cells in the brain, followed by the liver, cochlea, and kidney. The magnetic interaction between the produced PCS (poly clustered superparamagnetic iron oxide) nanoparticles and MSCs may increase stem cell delivery effectively. In a variety of experimental models examining cell responsiveness in vitro and in situ, the potential use of SPION to increase the spatial control of stem cells by the application of magnetic fields was examined. The application of the magnetic field has significantly increased the number of cells that migrate toward the area of magnet application in vitro. However, in in vivo settings the quantification of the effect of magnetic field was yet solved.

Conclusion

The visualization of delivered MSCs, as well as quantitative and qualitative analysis of the processes, will contribute in the formulation of an assessment system for a powerful delivery system in the inner ear, which will aid in the treatment of hearing loss

Introduction

Multipotent mesenchymal stem cells (MSC) are plastic-adherent, resemble fibroblasts in appearance, form colonies in vitro, and can differentiate into osteoblast, chondroblast, and adipocyte[1]. Owing to their multipotency, MSCs enable structural and functional repair of injured tissue. The migratory characteristics of MSCs and the stem cell's simple access to the target organ make it a potential therapeutic agent[2]. Homing is the ability of the circulating stem cells or exogenously administered stem cells to locate and enter environmental niche[3]. MSCs have the critical capacity to recruit and home stem cells and progenitor cells to the site of an injury in order to facilitate the regeneration of damaged tissue. MSCs express several cell-surface receptors, including some chemokine receptors, that mediate various aspects of homing. Despite this, however, MSCs do not express all critical chemokines and have low expression levels of other crucial adhesion receptors. These adhesion receptors regulate the anchoring and rolling of circulating cells on the activated vascular endothelium. In addition, MSCs do not express all essential chemokines[4]. Therefore, the development of additional ways to improve the homing phenomena of the MSC is essential in order to enhance the efficiency of stem cell delivery to the area of interest. Ototoxicity is a drug or chemical induced injury in the inner ear that leads to functional hearing loss or vestibular disorders[5]. The hair cells are the sensory cells located in the inner ear[6], they possess small potential for regeneration making their injury permanent[7]. Up to 33% damage has been documented in adult patients taking aminoglycoside antibiotics, and 10% of those patients have ototoxicity, with a 3% probability that the damage will be permanent, currently there is no available treatment for ototoxicity. Research on the application of stem cells in the field of inner ear disorder has been increased due to its capability to home through the complicated inner ear structure which consists of several barriers and repair the cochlear damage[8–11]. However, the drawback in the stem cell homing to the inner ear hair cell lies on the low efficacy of homing phenomenon, therefore an assisting strategy is needed to increase the delivery of the stem cells to the inner ear. The stem cell response to the injury involves the local paracrine signaling such as chemokines, cytokines, growth factors and prostaglandins[12]. The chemokine signals regenerated from the hair cell injury is not enough to home the stem cells to the inner ear due to its anatomical structure and location. To assist and enhance the homing of stem cells, nanoparticles and magnetic field has been utilized in various studies[13].

The superparamagnetic iron oxide nanoparticles (SPION) have been labeled in the stems cells and utilized as a magnetic resonance imaging (MRI) contrast agent and also as a tool for monitoring the migration of injected stem cells [14]. When exposed to magnetic field they induce magnetism, therefore, they have been applied in stem cell research in assisting tracking and magnetic manipulation.

In this study, Kanamycin-Furosemide induced ototoxic mouse model was utilized in determining the efficacy of the SPION labeled MSCs. The SPION-MSCs are injected in the middle ear cavity and are intended to target the inner ear wherein the auditory hair cells are located. The chemical signaling from the injured area is not enough to home stem cells to the anatomically complicated area such as the inner ear, therefore, to overcome the drawbacks of chemotactic homing, polyclustered superparamagnetic iron oxide nanoparticle (PCS nanoparticle was developed labeled in the MSC to magnetically assist and

enhance the homing phenomenon in the inner ear. The findings in this research will further support and enable the targeted delivery of MSC into inner ear and also overcome the obstacle in delivering the stem cells to the anatomically complex area for therapeutic use.

Methods

PCS Nanoparticles manufacturing and characterization

PCS nanoparticle synthesis

PCS nanoparticles were synthesized by using the following: poly (D,L-lactide-co-glycolide) (50:50, MW 38,000–54,000) (PLGA, Sigma-Aldrich, USA), iron oxide (II, III), magnetic nanoparticles solution (SPION, Sigma-Aldrich, USA), and 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide hydrochloride (EDC, Thermo-fisher scientific, Massachusetts, USA). N-hydroxysuccinimide (NHS) was used as a linker between the fluorescent molecule. Cy5.5 (Cyanine 5.5 amine, red; Lumiprobe Co., Hannover, Germany) was synthesized for detecting nanoparticles in the cells.

PCS nanoparticles, termed as PLGA-Cy5.5, were synthesized by EDC-NHS coupling; 330 mg of PLGA was dissolved in DMSO, with 60 mg of EDC, 132 mg of NHS, and 9.9 mg of Cy5.5, and incubated for 24 h. The nanoparticle mixture was extensively dialyzed (MW cutoff = 10 k) with deionized water to remove excess EDC, NHS, and Cy5.5. The resulting PLGA-Cy5.5 particles were freeze dried to obtain powdered samples

PCS Nanoparticles characterization

PCS nanoparticles are structured with a polymeric shell consisting of 10 nm sized SPIONs in the core, were prepared using an oil-in-water emulsion method. 1 mg of PLGA-Cy5.5 was dissolved in acetonitrile at 25°C and to this solution 0.1 mL of SPION (5 mg/mL) solution was added drop wise in 3 mL of deionized water. The vial was vortexed for 5 min, followed by 3 min of sonication. The mixture was stirred at room temperature for 6 h. The nanoparticles were further purified using an ultra-centrifuge and stored at 4°C until further use.

The size and surface zeta potential of the nanoparticles were obtained by dynamic light scattering (DLS) (Malvern, Zetasizer-ZS90). TEM measurement was performed to analyze the SPION core. Samples for TEM imaging were prepared by the drop casting over a carbon grid.

Hedayati's method[15] was applied to quantify the Fe ion concentration of the PCS nanoparticle solution. Briefly, for measuring the concentration of the Fe (iron) ion, acetate buffer, ferene-s solution, working buffer, and working solution were made. A total of 7.7 g of ammonium acetate (Fisher cat. No. A637-500, FW = 77.08) was added to 20 mL of glacial acetic acid (Fisher cat. No. A38-212) and mixed with DI water, to a final volume of 50 mL, to prepare the acetate buffer. The ferene-s solution was prepared by adding 0.5 g of ferene-s (Sigma cat. No. P4272, FW = 494.37) to 2 mL of DI water. To make the working buffer, 400 mg of L-ascorbic acid (Sigma cat. No. A92902-100, FW = 96.12) was added to 2.2 mL of acetate

buffer. Finally, 2 mL of working buffer, 0.1 mL of ferene-s solution, and 7.9 mL of DI water were mixed to make the working solution. A total of 50 µL of PCS nanoparticle solution was mixed with 950 µL of working solution.

Commercially available nanoparticle fluidMAG-CMX (Chemicell, Berlin, Germany) with similar physical characteristic with the lab synthesized nanoparticle has been purchased to compare its properties and evaluate the efficacy of PCS nanoparticles.

PCS nanoparticles labeling in MSC

Mouse bone marrow derived MSCs tagged with green fluorescent protein were cultured under 37°C and 5% CO2 in complete culture medium with MEM (Hyclone, UT, USA), supplemented with 10% FBS (Gibco, NY, USA), 1% penicillin/streptomycin, and amphotericin B. Labeling of the MSCs with the nanoparticles was performed with laboratory synthesized, 100 nm polymeric clustered superparamagnetic iron oxide nanoparticles (PCS nanoparticles). Adherent cells were incubated with 40 µg/mL PCS nanoparticle suspended media for 24 h.

Assessment of Viability in PCS nanoparticle labeled MSC

Cell proliferation and viability were quantified by using EZ-cytox cell viability assay kit (Daeil Lab., Seoul, Korea). Cells were seeded in a 96-well plate and were labeled with PCS nanoparticles in concentrations of 10, 20, and 40 µg/mL and absorbance were measured with a spectrophotometer, at 450 nm.

Evaluation of MSC Differentiation

The adipogenic and osteogenic differentiation of mouse bone marrow MSCs were performed using the cell differentiation kit (Mouse Mesenchymal Stem Cell Functional Identification Kit; R&D Systems, Minneapolis, MN, U.S.A.) according to the manufacturer's instructions.

Adipogenesis

For the adipogenic differentiation, 3.7 × 104 cells of MSCs were seeded into the coverslip in each well of a 24-well plate and cultured overnight at 37°C in 5% CO2. The adipogenic differentiation medium (0.5 mL) was composed of α-Minimal Essential Medium (MEM) with 10% FBS, 1% antibiotic-antimycotic (Penicillin 10,000 units/mL, streptomycin 10,000 µg/mL, and Fungizone® (amphotericin B) 25 µg/mL diluted 1/100) (Thermo Scientific) supplemented with 5 µL of adipogenic supplement (containing hydrocortisone, isobutylmethylxanthine, and indomethacin). The medium was replaced every 3 days for 14 days.

Osteogenesis

Osteogenic differentiation was evaluated, 7.4 × 103 cells of MSCs were seeded into fibronectin coated coverslips in a 24-well plate and incubated overnight at 37°C in 5% CO2. The next day, the cells were cultured in osteogenic differentiation medium (0.5 mL) composed of α-MEM basal medium containing 25 µL mouse/rat osteogenic supplement (containing ascorbate-phosphate, β-glycerolphosphate, and

recombinant human bone morphogenetic protein (BMP-2)). The medium was replaced every 3 days for 21 days. The adipocytes were identified by staining with an anti-mFABP4 antibody (R&D Systems). The osteogenic cells were identified by staining with an anti-mOsteopontin antibody (R&D Systems).

Flow Cytometry Analysis of PCS Nanoparticle Labeled MSC

Green fluorescence protein (GFP) tagged MSCs were cultured in 60mm culture dish with initial seeding concentration of $3x10^5$cells/dish. 40ug/ml of PCS nanoparticles are added. 24h post labeling the cells were washed with PBS, detached with EDTA and centrifuged at 500G and resuspended in PBS. Samples are analyzed using FACS Aria3 flow cytometer (Becton Dickinson, San Jose, CA, USA). The data were analyzed using FACS Diva software (version 6.1.3).

Immunofluorescence Imaging of PCS nanoparticle Labeled MSCs

GFP tagged MSCs were seeded onto 4-well cell culture plates at a density of 5×10^3 cells/well. 24 h after the culture media was replaced with fresh media and PCS nanoparticles were added. 24h post labeling the cells were washed twice with PBS and fixed in 10% paraformaldehyde and stained with 4',6-diamidino-2-phenylindole (DAPI). The samples were observed by confocal microscopy (Carl Zeiss Microscopy GmbH, Jena, Germany), images were analyzed using ZEN lite ver. 2.3.

Transmission Electron Microscopy of PCS nanoparticle Labeled MSCs

The cells were fixed in 2.5% glutaraldehyde for 2 h at 4°C and the specimen made to solidify on 2% agar. Samples were post fixed in 1% osmium tetroxide (OsO_4) after being washed with 0.1 M cacodylate buffer. The dehydration steps were performed using 50–100% ethanol and embedded onto Epon resin. Ultrathin sections were cut using an ultra-microtome and stained using uranyl acetate and lead citrate. All samples were observed using the transmission electron microscope.

Attraction of PCS nanoparticle labeled cells to static magnetic field

Green fluorescent protein tagged MSCs were seeded in a 1-well culture dish and the cells were left to attach; 40 µg/mL of nanoparticles were added in the culture medium to label the cells for 24 h. After labeling, the cells were PBS washed at least twice and the culture media was added. A 3 mm NdFeB magnet, with magnetic force of 0.34 T, was attached at one side of the culture slide and the cells were magnetically attracted for 24 h. Magnetically attracted cells were fixed in 4% paraformaldehyde for 15 min and washed with PBS twice. The nuclei were counterstained with DAPI (Fluoroshield™ with DAPI, Sigma-Aldrich, Co., MO, USA), a coverslip was placed on the samples, which were then left to dry for 24 h. The samples were observed under the fluorescence microscope. Fluorescence images were taken from the culture slide.

Chemotactic and magnetic migration assay

Migration assays were performed by using Costar migration chambers (Transwell® 6.5 mm diameter chambers, 8.0 μm pore size, 24-well, Kennebunk ME, USA). PCS nanoparticle labeled MSCs were plated at a concentration of 2×10^4 in the upper chamber of the well, supplemented with media containing 1% FBS. Complete culture media with the chemoattractant, SDF-1, was placed in the lower chamber for 24 h, and 5mm cubic neodymium were placed at the bottom of the culture plate to observe the migration of MSC with or without influence of magnetic field. The membranes were fixed with PFA and stained with crystal violet and observed under the light microscope.

Synthesis of Static magnetic field

Simulation of Dual Permanent Magnet

To obtain the simulation result, we create 2 permanent magnets (2.54 x 1.27 x 0.64 cm), with the remanence ~ 0.29 Tesla across their width. The two magnets rotated inward at 30 degrees. Step 1: Create 3D model with study AC/DC > Magnetic Fields, No Currents > Magnetic Fields, No Currents (mfnc) and choose General Studies > Stationary. Step 2: GEOMETRY; first create 2 blocks with match size for 2 magnets then create 1 block for air environment. Step 3: Assign material for each block. Step 4: Assign Magnetic Flux Conservation for each magnet with calculated magnitude. Step 5: Mesh Step 6: Study. Step 7: Add 3D Plot Group; slice to set the magnitude of magnetic flux density, 2. Arrow volume to see the direction of magnetic field (If the arrow is proportional to magnitude of B, it will be hard to observe so we just choose normalized mode to check the direction). Steamline to obtain magnetic field lines (magnetic flux density).

Streotaxic Magnetic Device

Two neodymium magnets with opposite poles were place inside a 1cm x 7mm metal cylinder with blunt ended conical head to generate high magnetic force. Tesla meter was used to measure the magnetic force. In a rectangular metal plate, streotaxic arms which allows free joint movement were designed and placed in four corners to facilitate animal experiment.

In vivo evaluation of stem cell delivery

Animals

C57BL/6J, five-week-old, male mice with body weight average 18–22 g were purchased from Daihan Biolink (DBL Co., Ltd, Eumseong-gun, Korea) to assess the delivery of PCS nanoparticle labeled stem cells after drug induced ototoxicity and hearing threshold was evaluated. The mouse was acclimated 1-week prior experiment in 12 h light/dark cycle with room temperature and was allowed free access to water and food. The study was performed in accordance with the Institute Ethics Committee of Yonsei University Wonju College of Medicine. The animal experiment protocol was approved by Institutional Animal Care and Use Committee of Yonsei University Wonju College of Medicine with approval number of YWC-190513-1.

Ototoxic Animal Model

C57BL/6J male mice were purchased from Daihan Biolink (DBL Co., Ltd, Eumseong-gun, Korea) and used to generate ototoxic animal model. Kanamycin sulfate 550 mg/kg (VWR life sciences, PA, USA) and furosemide 130 mg/kg (Lasix, Handok, Korea) were administered via subcutaneous and intraperitoneal injection, respectively.

Auditory Brainstem Response (ABR) Recording

The mice were anesthetized with 100 mg/kg ketamine (Yuhan, Seoul, Korea) and 10 mg/kg xylazine (Rompun, Bayer, Ansan, Korea) by intraperitoneal injection prior ABR recordings. The anesthetized mice were tested in a sound attenuating chamber with a built-in Faraday cage. An isothermal pad was used to maintain the body temperature of the test subject. TDT RZ6/BioSigRZ system (Tucker Davis Technologies, Alachua, FL, USA) was used for stimulus generation, data management, and ABR collection.

Twelve-millimeter-long, gauge 27 subdermal needle electrodes (27GA 13 mm, Rochester Electro-Medical, USA) were used to record the ABR (Fig. 1A). One channel was recorded: active electrodes were placed in the vertex; reference electrode was placed axial to the pinnae, which is the same side as the stimulus delivery; and ground electrode was placed on the contralateral side. The electrodes were connected with the low impedance head stage RA4LI, TDT) that interfaces with the TDT amplifier.

Acoustic stimuli were generated by auditory processor (RZ6, Tucker Davis Technologies, Alachua, FL, USA). The stimulus signal and signal response data were acquired by automated processing through BioSigRZ software installed on the PC. Stimuli were delivered in a closed field setting by a magnetic speaker (MF1, TDT, Alachua, FL, USA) with a PVC tubing and a conical cap inserted into the subject's ear.

Evaluation of ABR Recording in Ototoxic animal model

ABR were recorded from the bilateral ears, 1 day prior to administration (Day-1) of the ototoxic drug and on days 3, 5, 7, 10, 14 post administration of the ototoxic drug. Prior to recordings, mice were anesthetized with a mixture of ketamine 100 mg/kg and 10 mg/kg xylazine. The stimuli were given at a maximum of 90 dB to a minimum of 10 dB for clicks. Ten decibel steps reducing the SPL to obtain the auditory thresholds. Using a pair of MF1 microphones and an etymotic research microphone, ABR recordings were measured. The stimuli were generated using TDT software.

Magnetic delivery of MSC-PCS administered by intrabullae injection

The mouse is anesthetized with 100 mg/kg ketamine (Yuhan, Seoul, Korea) and 10 mg/kg xylazine (Rompun, Bayer, Ansan, Korea). The mouse is placed in lateral supine position to easily locate the bullae. Incision is made at the posterior auricular area 5mm below the pinnae. Submandibular gland is exposed and removed to secure visual field. Bullae was visualized by removing surrounding tissues, tap dry with cotton swab to facilitate puncturing the bone. Puncture bullae with 0.1mm micro drill, inject PCS nanoparticle labeled MSC using Hamilton syringe with 32-G needle and seal with bone wax. Cell overflow

or leakage was prevented by placing the subject lying contralateral to the infusion site. Transplantation events required 40–50 s each. In the magnetic field exposed group, the mice were placed in the left lateral position. Two permanent magnet facing opposite pole with magnetic force of 530mT facing each other was placed 2cm above the left bullae area for 40 min. ABR recording were recorded on 7, 14, 21 days post MSC-PCS administration to evaluate the efficacy of stem cell delivery in the ototoxic mouse and to compare threshold difference in MSC-PCS delivered left ear versus control right ear.

Evaluation of otoprotective effect of MSC-PCS by assessment of inflammatory cytokines

Reverse transcriptase PCR was performed to quantify the nanoparticle labeled cells in different tissue samples. Total RNA from the cochlea was extracted using TRIzol Reagent (Invitrogen, Carlsbad, CA, USA). A total of 1 µg of RNA was reverse transcribed using ReverTra Ace® qPCR RT master mix with gDNA remover (Toyobo, Osaka, Japan) and cDNA was used as a template for PCR amplification. Reverse transcriptase PCR was performed using Applied Biosystems SimpliAMP thermal cycler to quantify the cytokine levels. Primers used in reverse transcriptase PCR were listed in Table 1.

Table 1
Mouse primer sequences used in reverse transcription PCR

Gene	Primer Sequence (5'-3')	Size (Bp)
β-actin	Forward: TCTCCAGCAACGAGGAGAAT	348
	Reverse: TGTGATCTGAAACCTGCTGC	
IL-1β	Forward: GCACTACAGGCTCCGAGATGAA	145
	Reverse: GTCGTTGCTTGGTTCTCCTTGT	
IL-6	Forward: CCGGAGAGGAGACTTCACAG	421
	Reverse: GGAAATTGGGGTAGGAAGGA	

Tissue Clearing of Cochlea

Mouse were sacrificed and transcardiac perfusion was performed with 50-100ml ice-cold PBS and 50 ml 4% PFA for fixation. Collect cochlea and immerse in 4% PFA for 24h at room temperature for fixation. Immerse samples in 20% EDTA (pH 7.0) at 37°C in a shaker for 2–3 days (depending on the degree of decalcification). Wash samples with water for at least 30 minutes to elute excessive EDTA. Decolorize with quadrol decolorization solution for 2 days and 5% ammonium solution at 37C at shaker. Place samples in gradient tB delipation solution for 1–2 days then tB-PEG for 2 days for dehydration. Immerse samples in the BB-PEG medium at 37C for at least 1 day for clearing. Immune staining should be performed post decolorization step ten proceed further steps.

Statistical Analysis

Statistical comparison was made using two-tailed unpaired student's t-test. Data analysis of all obtained data was performed with GraphPad Prism software (version 8, GraphPad Software, San Diego, CA). Data are presented as the mean ± standard error of the mean. P values less than 0.05 were considered significant for single and multiple comparisons.

Results

Physicochemical Characterization of PCS Nanoparticles

The intrinsic properties of the nanoparticles have direct interaction with cellular uptake and biocompatibility. Nano-sized PCS nanoparticles (PSCs) were prepared by the bottom-up method, in oil-in-water emulsion as described in our previous study[16]. The schematic representation of PCS nanoparticle is described in Fig. 1A. PCSs have two components, i) an oleic acid-coated SPION core, which confers high stability to the nanoparticle, and ii) a PLGA-Cy5.5 polymer shell, which enables the nanoparticle to interact with the aqueous phase and increases the biocompatibility of the nanoparticle with the fluorescent moiety.

The particles' suitability for further in vitro experiments was determined by measuring their size and surface charge. The physicochemical properties of PCSs were measured by Zetasizer-ZS90. The hydrodynamic diameter was calculated to be 114 nm, with 0.162 poly-dispersity index (PDI), using dynamic light scattering (DLS) (Fig. 1). The surface ζ-potential was − 29.6 mV, under aqueous conditions. The PLGA-Cy5.5 shell provides high biocompatibility and biodegradability.

Endocytosis of PCS nanoparticle

PCS nanoparticles internalized in MSCs were detected through Prussian blue staining, ferric ions inside the cells were observable in blue granules (Fig. 2A). Total amount of ferric ions was analyzed through ferene-s assay and the concentration of PCS nanoparticles in cells increased in accordance with the increasing concentration treated (Fig. 2B). TEM imaging of PCS nanoparticle labeled MSCs to localize the internalized nanoparticles (Fig. 2C).

Further detection of internalized PCS nanoparticles was observed by detecting Cy5.5 through confocal microscopy (Zeiss, LSM800). Green fluorescence indicates MSCs tagged with GFP; nuclei, stained with 4',6-diamino-2-phenylindole (DAPI), can be observed in blue; and red fluorescence shows a PCS nanoparticle tagged with Cy5.5 (Fig. 2D). Flow cytometry analysis was performed to determine the labeling efficacy according to labeling time, the results shows that the PCS nanoparticle labeled cells increases with prolonged labeling time, the peak time for labeling was at 24h (Fig. 2E).

The PCS nanoparticles are evaluated as compared with fluidMAG-CMX (Chemicell, Berlin, Germany). It has polymer matrix of carboxymethyldextran which is similar with PCS nanoparticles with average hydrodynamic size of 100nm. fluidMAG-CMX nanoparticles are labeled with different concentrations and

total iron content in MSCs were determined through ferene-S analysis. In terms of internalization of the nanoparticles the PCS nanoparticles had greater concentration of internalized nanoparticles compared with the fluidMAG-CMX nanoparticles when given in same concentration. Therefore, this result indicates that the lab synthesized PCS nanoparticles efficiently labeled in the cell in comparison with the commercially available nanoparticle (Fig. 3).

Cytotoxicity and Differentiation of PCS nanoparticle labeled MSC

Cells were dose dependently labeled with nanoparticles and the viability was assessed through spectrophotometry. The highest concentrations (80µg/mL) of PCS did not have a significant effect on cell viability up to 48 h, however toxicity was observed 72 and 96h post labeling at concentrations from 50 to 80 µg/mL. cells labeled with 40 µg/mL of nanoparticles had more than 90% viability. Based on these findings, the optimum concentration for MSC labeling was set at 40µg/mL (Fig. 3A)[17]. Furthermore, the preservation of the stemness in PCS labeled MSCs were evaluated through its differentiation into adipocytes and osteocytes (Fig. 3C).

Magnetic attraction enhanced migration PCS nanoparticle labeled MSC

The Fe in the PCS nanoparticles core possesses the property of magnetization when an external magnetic field is applied. MSCs labeled with the PCS nanoparticles were attracted with a magnet to determine the enhancement of migration capacity. MSCs were cultured in a 1-well culture dish, labeled, and a magnet was attached to one side. After 24 h incubation and 48 h incubation, the cells were fixed and DAPI stained, and fluorescence images were captured at 1 mm intervals from the magnet. total of 50% of cells adhered to 0–5 mm distance from the magnet, 30% adhered to 5–10 mm distance from the magnet, and 10% adhered to 10–15 mm distance from the magnet (Fig. 4). Thus, an increased number of cells was observed in the area near the magnet; the migration of the PCS nanoparticle labeled cells could be manipulated by placing an external magnet. However, this experimental had limitations due to the distance of the magnet and cell which could interfere with the degree of magnetic field influence. Thus, alternative experimental setup was established by directly the magnet under the 35mm culture dish. Crystal violet staining was performed to visualize the location of the MSCs. Increased cell density around the magnet area was observed in PCS nanoparticle labeled MSCs (Fig. 4D).

Customized Static Magnetic Device Development

Specialized static magnets were devised and constructed for use in experimental settings. To generate the appropriate magnetic force, two magnets are positioned oppositely within a metal cylinder and one end is designed with a conical form to emit magnetic flux over a long distance; the final product is a cylinder magnet measuring 1cm x 7mm in diameter with a magnetic flux density of 535.2mT. (Fig. 5A). Streotaxic arms are intended to aid in the placement of the magnet at a precise point.

Magnetic Push Flux density

The fundamental idea behind this technique is to use two permanent magnets to push our ferro material particles away utilizing the magnetic force that is discussed in the major theory section. As seen in Fig. 5, a single magnet generates a magnetic field line all the way around its length. Our two magnets precisely overlap their magnetic fields with another magnet whose pole is inverted, causing their magnetic fields to cancel out at the node point X but not in the surrounding region. The H vector is therefore equal to zero at location X. Due to the fact that the direction of the magnetic force acting on the particles is the same as the gradient vector of magnetic intensity H, or magnetic flux density B, the force moves away from point X, where H = 0, to the region surrounding that point, where H = 0.

We utilized the AC/DC Module of COMSOL Multiphysics (version 5.5) to create the same set up with two magnets (2.54 x 1.27 x 0.64 cm), each of which is assigned as a neodymium rare earth material with magnetic susceptibility _mag73 and a magnetization M = 300 (kA/m) travels through each magnet. This allowed us to both compute and observe the phenomenon. Due to the magnetization, a residual flux density of 0.29 Tesla (Br) will be created throughout the width of the magnets. As can be seen in Figure X, two magnets are rotated at an angle of thirty degrees in the opposite direction. The particle is a sphere with a diameter of X, located X cm from the point X. The environment is set to air, which has a magnetic susceptibility that is very close to zero (3.6 x 10 − 7). Figure X presents the findings of the study.

B: **Magnetic flux density**/ Magnetic induction/ Magnetic field (ambiguous) – Unit: T = Wb/m^2

H: **Magnetic field intensity**/ Magnetic field strength/ Magnetic field/ Magnetizing field – Unit: A/m

M: **Magnetization vector –** Unit: A/m

Relation between parameters:

$$B = \mu_0(H + M)$$

$$M = \chi H$$

With χ is the volume magnetic susceptibility and $\mu_0 = 4\pi.10^{-7}$ H/m is the permeability of vacuum.

In a current free area, we have

$$\nabla \times H = 0$$

Thus

$$\nabla \bullet B = 0$$

The Magnetic moment (*m*) of the particles is given by [18]

$$\overrightarrow{m} = \frac{3\chi}{\chi + 3} V \overrightarrow{H}$$

With V is the volume of the spherical particle.

The force on particle can be calculated by

$$F = \nabla (m \bullet B)$$

The force applied on a single particle with diameter *d* can be present by the gradient and the magnetic field intensity at its location [19, 20]

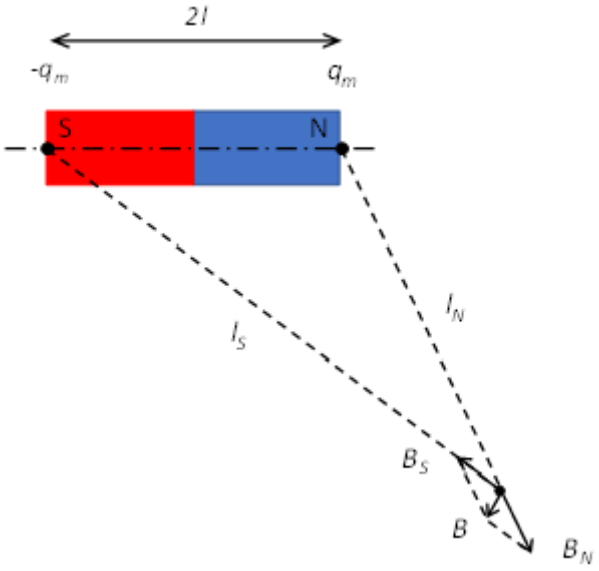
$$\overrightarrow{F} = \frac{2\pi d^3}{3} \frac{\mu_0 \chi}{1 + \frac{\chi}{3}} \nabla \left(\left| \overrightarrow{H} \right|^2 \right)$$

Moreover, the magnetic flux density at a location can be determined by the pole strength q_m (A.m) and distance from particle to North and South poles of the magnet (l_N, l_S)

$$\overrightarrow{B} = \overrightarrow{B_N} + \overrightarrow{B_S}$$

$$\overrightarrow{B_N} = \frac{\mu_0}{4\pi} \frac{q_m}{{l_N}^2}$$

$\overrightarrow{B_S} = \frac{\mu_0}{4\pi} \frac{-q_m}{{l_S}^2}$



$$\overrightarrow{m_{mag}} = q_m 2l = \frac{1}{\mu_0} B_r V_{mag}$$

Where:

m_{mag} is the magnetic moment of the magnet

B_r is the residual flux density (remanence)

V_{mag} is the volume of the magnet (in m3)

In Vitro Analysis on Magnetic Delivery

It has been determined that the concept of applying magnetic push flux density can be significant, and a series of simulations have been run in order to validate the probability that it can have an effect. The positioning of the magnet was determined by the outcomes of the simulation. The initial test of the magnetic push prototype was carried out with the assistance of stereotaxic arms, and the angle of two magnets was modified in order to provide the magnetic push force. An Eppendorf tube was used to contain a metal ball, and data regarding the motion of the metal ball was afterwards recorded (Fig. 5C). The magnetic pull and push forces were applied during the transwell migration assay. Both the magnetic pull and the magnetic push only produced findings that were almost identical, which suggests that the PCS nanoparticle labeled MSCs respond to both types of magnetic forces (pull and push). In contrast, the addition of the chemo attractant SDF-1 to the lower well caused a twofold increase in the number of cells that moved to the lower well (Fig. 5D). This indicates that the magnetic push force is more effective than the magnetic pull force.

In Vivo Analysis on Magnetic Delivery

An evaluation of the magnetic push system in an in vivo setting has been conducted out. In the ototoxic mouse model, PCS nanoparticle labeled MSCs were delivered into the bullae of the ear through intrabullae injection. PCS nanoparticle tagged MSCs can be generated by incubating 40 ug/ml of nanoparticles in the medium for twenty-four hours. Cells at a concentration of 5×10^5 in a volume of 5 ul were prepared for injection into the bullae. The animal models are grouped into three groups: a. the control group (n = 30), which received solely MSC; b. the magnetic push group (n = 30); and c. the magnetic pull group (n = 30). Following the treatment of the ototoxic drugs kanamycin and furosemide for a period of seven days, the PCS nanoparticle labeled MSCs were injected into the bulla of the mouse model. In order to provide a push force, two reciprocating magnets inclined at 45 degrees were positioned 2 centimeters above the bullae while the mouse was positioned on its lateral side. Magnets are positioned in the region of the mouse known as the vertex in order to impart a pulling force. The mouse model was exposed to a magnetic field for a period of forty minutes, alternating between the two different magnet application settings. Hearing threshold was evaluated using ABR click stimuli on days 7, 14, and 21 days (14, 21, and 27 days after treatment with the ototoxic drug) following MSC transplantation. The MSC + PCS group had a 20% efficiency, while groups B and C both achieved a 40% efficiency. Statistical analysis demonstrated that the MSC + PCS group that had push magnetic flux showed significant results on day 27 after MSC injection (Fig. 6).

PCS labeled MSCs Reduce Inflammatory Cytokines

The mRNA levels of inflammatory cytokine were evaluated using polymerase chain reaction in order to verify the effect that led to the recovery of hearing threshold in PCS nanoparticle labeled MSC that was injected to an ototoxic mice model. In this study, samples of mouse cochlea were taken, and levels of pro-inflammatory cytokine were evaluated. When compared to the group that did not receive PCS, the ototoxic mice group that received PCS nanoparticle labeled MSC injection had significantly reduced levels of the pro-inflammatory cytokines IL-1 and IL-6 (Fig. 6D).

PCS nanoparticle labeled MSC observation in Inner Ear

The confirm the delivery of the PCS nanoparticle labeled MSCs into the inner ear, the cochlea sample was collected from the mouse model post-delivery of PCS nanoparticle labeled MSCs with magnetic attraction. The cochlear samples are decalcified, and the surrounding bones are removed, in order to explant the organ of corti of the cochlea, which contains the auditory hair cell. Under a bright-field microscope, tissue samples are fixated and stained with Prussian blue to evaluate the accumulation of iron. At the end of the process, the iron accumulation in the organ of corti area can be observed clearly under bright field microscope (Fig. 6A). The clearing of cochlear tissue samples was processed to identify the presence of PCS nanoparticles labeled MSCs. Confocal microscopy of the cleared cochlear samples was done, however the localization of the MSCs was not completely processed. Further investigations are necessary to specify the migration of the MSCs.

Discussion

Stem cell therapy in ototoxic hearing loss

In the realm of regenerative medicine, the mesenchymal stem cells have been the subject of increasing research. The efficacy of treatments based on stem cells is mostly dependent on the efficient delivery of cells to the target region, where the cells will indeed release substances that have immunomodulatory and regenerative capabilities. However, several of the target locations are in difficult-to-reach regions, such as the cochlea and the vestibular system of the inner ear. There have been many different approaches proposed to improve the homing and engraftment of MSCs as a therapeutic tool in ototoxicity[16, 21–23], delivery of the stem cells have enhanced the ability to restore hearing. Increasing the biological effects of lipid rafts, altering the expression and function of homing molecules (the CXCR4/SDF-1 axis), regulating the metabolism of MSCs, and improving the availability of chemotactic factors for MSCs are some of the strategies that have been utilized[13, 23, 24]. In our previous study we have demonstrated MSCs that were labeled with nanoparticles and had higher expression of the CXCR4 receptor, which is a chemokine receptor located on the surface of MSCs, demonstrated greater homing ability in animal models[16, 23]. However, chemotaxis is not enough to guide stem cells home to the inner ear to perform therapeutic effect, anatomic impediments to absorption at the round window membrane (RWM), drug loss through the Eustachian tube, and varied or unknown pharmacokinetic characteristics of

drugs currently administered via this route are among potential downsides of local delivery of therapeutic agents. Due to the complex and unique anatomy, the diagnosis and treatment of inner ear diseases has been a dilemma due to the limitation in the access. Blood-perilymph barrier, blood-endolymph barrier, perilymph-endolymph barrier, middle ear barriers RWM and oval window annular ligament, are the anatomical barriers that limits the access to the inner ear.

The application of SPION as a homing facilitator

To overcome the drawbacks of chemotaxic homing of stem cells to the inner ear, superparamagnetic nanoparticles have been synthesized in accordance with the factors that could affect the efficacy of labeling in the cells and directly manipulate the migration through magnetic attraction. Finding an efficient delivery approach requires first determining the fate of internalized SPIONs. The essential factor about the PCS nanoparticles is that the PLGA coating made them more physiologically stable. The surface potential of the PCS nanoparticle was − 29.6 mV, which made them more compatible with the MSC since the surface of the cells is also negatively charged and does not interfere with the viability of the cells. The magnetic force is directly proportional to the absolute amount of Fe used in each experiment, which has implications for the structural characteristics of nanoparticles. Through series of experiments, we have observed that the cellular uptake of nanoparticles in concentrations more than 40 ug/mL did not enhance the labeling efficacy.

As a result, nanoparticles and a static magnetic field have been added as a means of directing the stem cells to the desired location in order to facilitate the delivery and retention of the MSCs, which will ultimately result in enhanced effects. The widespread potential applications of SPIONS in the biomedical field contributed to the growth of interest in the technology. Numerous researches have been conducted to examine the nanoparticles for their potential application in delivery of drug [25, 26].

Static magnetic field guided delivery of Stem cells to target area

These research on the application of magnetic flux density on the administration of PCS nanoparticle labeled MSCs gave a basis on the effective delivery of stem cells to the inner ear. The static magnetic field has been applied in the experiment setting since it generates desirable force to manipulate the movement of PCS nanoparticle loaded MSCs. The static magnetic field is safe and easy to apply, this would also further increase the potential use in the clinical setting. In the in vitro experiment setting the static magnet was placed on one end of the culture plate and allowed the MSC-PCS to attract towards the magnet, within 24 h the cells were able to migrate towards the magnet for 5-10mm, however, in vivo experiment setting did not reflect in vitro results. Therefore, static magnetic field exposure in the animal experiment was modified to generate the expected results. Studies have shown that magnetic fields placed in opposite directions, when the two magnets precisely overlap magnetic field, they cancel at the node point hence creating a zero-force area. Beyond the zero-force area, an outward magnetic force is created allowing magnetically push the iron oxide nanoparticle, since the MSCs were internalized with the

PCS nanoparticle their movement was able to be manipulated by the external magnetic flux. These results would further support as the delivery option of SPION labeled stem cells to the inner ear as well as those area which are located in anatomically complicated area. In spite of this, there are a variety of factors that need to be taken into consideration when engaging with the static magnetic field. It is likely that the ability to direct the migration of MSCs to the target site may lead to an enhancement in the processes of drug delivery and regeneration.

Otoprotective effect of the MSC-PCS

Restoration of hearing as a consequence of administration of PCS nanoparticles labeled MSCs is another significant finding of this study. The aminoglycoside induced ototoxicity is known to be generated by the disruption of mitochondrial protein synthesis, overactivation of glutamatergic receptors (N-methyl-D-aspartate), and formation of free radicals[27]. The free radicals generated by the aminoglycoside will further cause inflammation in the hair cells as well as the neurosensory cells in the inner ear which will subsequently lead to cellular injury and apoptotic cell death[28]. Consequently, in this study we expect that hearing threshold difference is likely if a significant number of stem cells are delivered to the targeted area. However, in the early stages of stem cell administration no significant changes in the hearing threshold were observed, and hearing restoration was observed 7 days post administration, this result may implicate that it affects the regeneration and restoration of hearing rather than protecting the hair cells from initial acute inflammatory response. The anti-inflammatory cytokines produced by the stem cells would also affect the depth of cellular injury by the aminoglycosides. The pro-inflammatory cytokines interleukin 1 beta and interleukin 10 levels has been investigated in the study; these cytokines are key mediator of the inflammatory response. PCR results revealed the levels of pro-inflammatory cytokines have significantly decreased when PCS nanoparticle labeled MSCs were delivered to the cochlea compared with those MSCs only. The SPION has heat-releasing properties known as the magnetic hyperthermia[29], the clinical hyperthermia involves the local temperature increase within the range of 43-47C, this phenomenon has been studied in the field of cancer research. The heat production of the nanoparticles would increase the production of heat shock protein (HSP) in the stem cells, they are released from the cell through exosome or extracellular vesicle release [29–31], the release of HSP70 which would protect and promote replacement the lost hair cells through differentiation of supporting cells.

Conclusion

Here, we have developed nanoparticles and magnetic device to deliver stem cells to the inner ear. In this study we evaluated the therapeutic effect of PCS nanoparticle labeled MSCs delivered to the inner ear and observe changes in inflammatory factors. In the future study to address the phenomenon on therapeutic effect of the stem cells, we will focus on the significant production of extracellular vesicle, release of the HSP70 and its effect on the protection and regeneration of the hair cells. Additionally, the stereotaxic magnetic device could be further developed optimized for inner ear application.

Abbreviations

ABR
Auditory Brainstem Response
CMX
Carboxymethyl dextran
DAPI
4',6-diamidino-2-phenylindole
GFP
Green fluorescence protein
HSP
Heat Shock Protein
MSC
Mesenchymal Stem Cell
MRI
Magnetic Resonance Imaging
NdFeB
Neodymium Iron Boron
PCS
Polyclustered Superparamagnetic Iron Oxide Nanoparticles
PFA
Paraformaldehyde
PLGA
Poly(lactide-co-glycoside)
RWM
Round window membrane
SPION
Superparamagnetic Iron Oxide Nanoparticles

Declarations

Ethics approval and consent to participate: The project entitled Enhanced homing capacity and biodistribution of superparamagnetic iron oxide nanoparticle labeled mesenchymal stem in ototoxic mouse model was performed in accordance with the Institute Ethics Committee of Yonsei University Wonju College of Medicine. The animal experiment protocol was approved by Institutional Animal Care and Use Committee of Yonsei University Wonju College of Medicine with approval number of YWC-190513-1 on May 20, 2020.

Consent for publication: Not applicable

Availability of data and materials: The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Competing interests: The authors declare no competing interests

Authors' contributions: YA: conception and design, collection and assembly of data, data analysis and interpretation, manuscript writing; SMC: collection and assembly of data; JP: collection and assembly of data; TH: data analysis and interpretation; JC: data analysis and interpretation; SL: Data analysis and review; DP: Data analysis and review; SH: Data analysis and review; TB: Data analysis and review; SHC: Data analysis and review; JK: Data analysis and review; YS: administrative support, collection and assembly of data, data analysis and interpretation, manuscript writing, final approval of manuscript.

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Figures

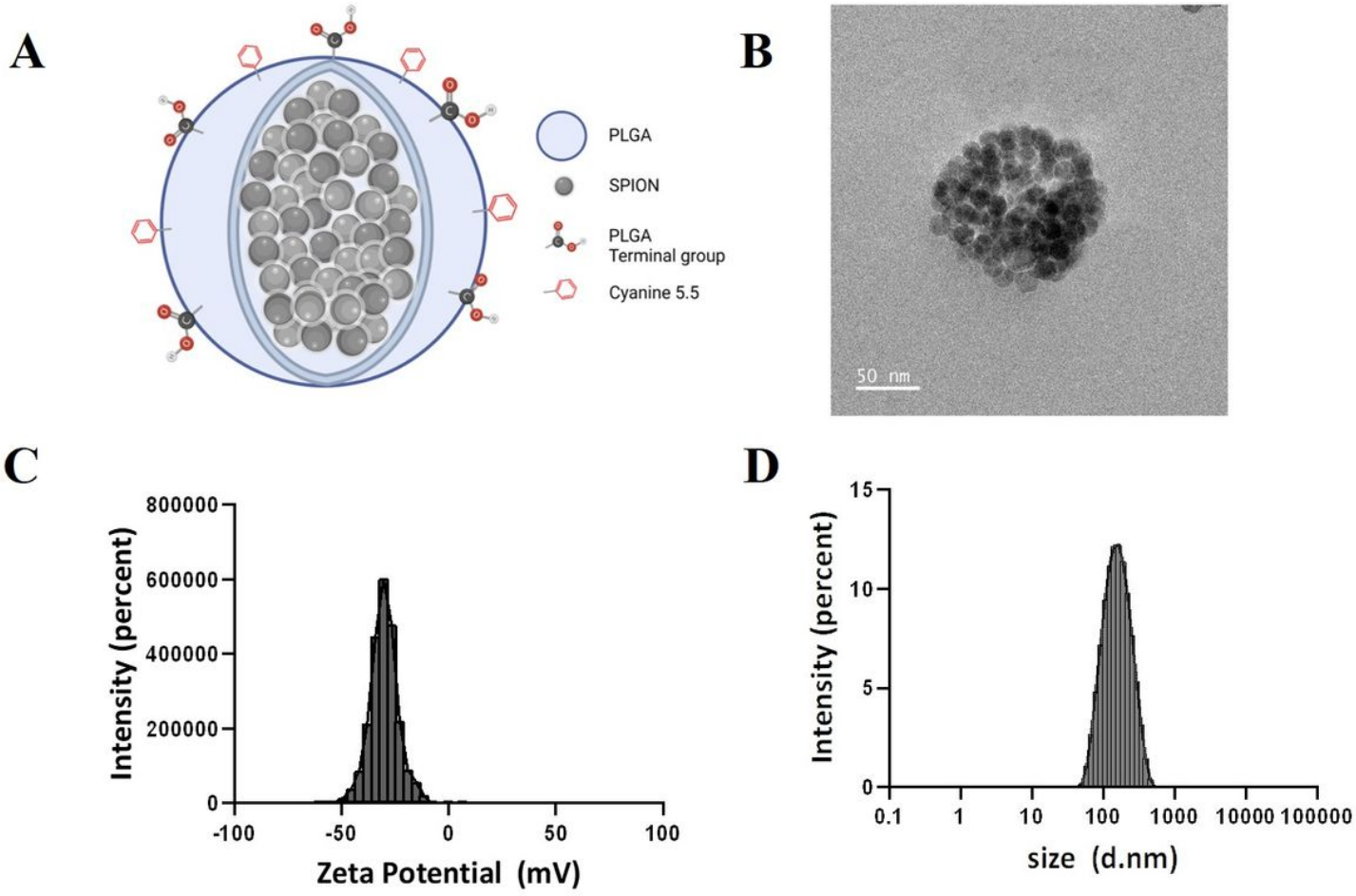

Figure 1

Physicochemical properties of PCS nanoparticle. A Schematic image of nanoparticle B TEM image of the PCS nanoparticle showing clustered SPION cores. C Surface zeta potential D hydrodynamic size of the nanoparticle

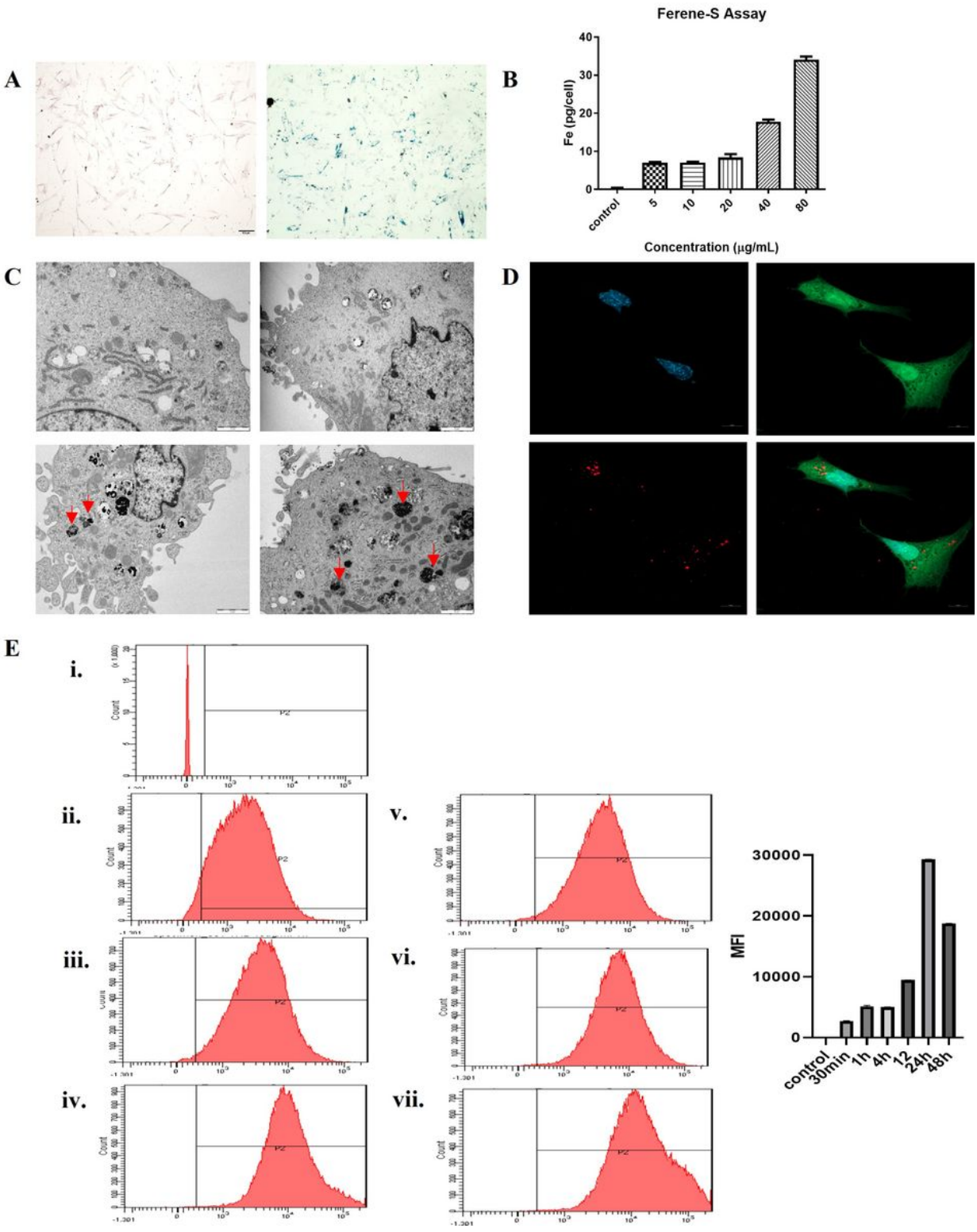

Figure 2

Endocytosis of PCS nanoparticles in MSC. A. Prussian blue staining B. Ferene-S analysis of PCS nanoparticle labeled MSC. C. TEM imaging red arrow indicates internalized nanoparticles. D. Confocal imaging, MSCs tagged with GFP observed in green, red fluorescence from Cy5.5 tagged in PCS nanoparticles. E. Flow cytometry analysis of PCS nanoparticle labeling in MSC.

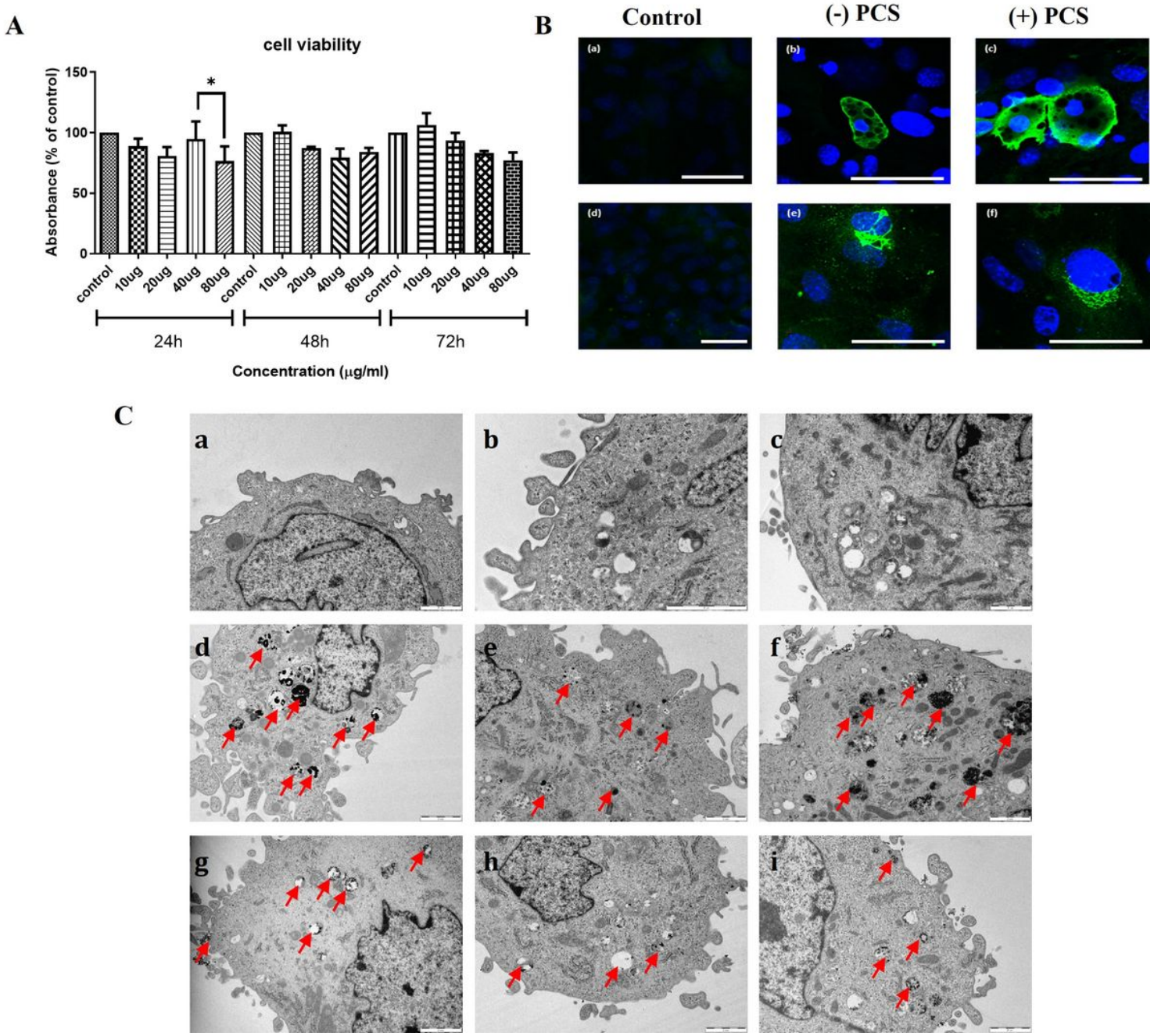

Figure 3

Cytotoxicity and Differentiation of PCS nanoparticle labeled MSCs. A. CCK-8 assay 24, 48, and 72h post PCS nanoparticle incubation with different concentrations. B. Evaluation of adipogenic and osteogenic differentiation of PCS nanoparticle labeled MSCs. C. TEM image of internalized PCS nanoparticles within the MSC (red arrow indicates nanoparticles); a-c MSC without nanoparticle labeling, d-e 40ug/ml PCS nanoparticle labeled MSCs, g-i 40ug/ml FluidMag-CMX labeled MSCs

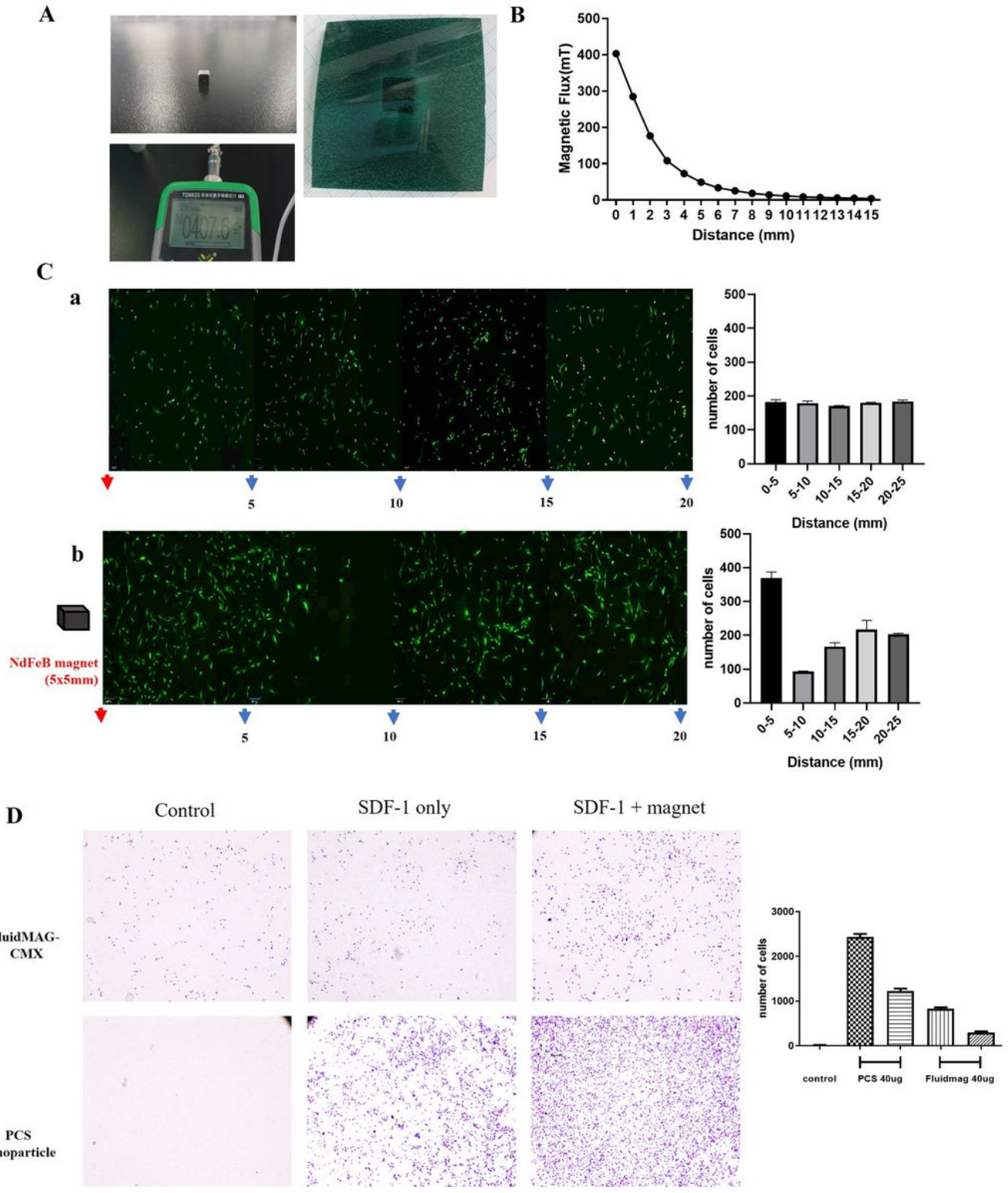

Figure 4

Assessment of Magnetic Properties and evaluation of cell response to chemotaxis and magnetic attraction. A. Cuboidal NdFeB magnet properties and magnetic force. B. Magnetic force displacement by distance C. Fluorescent image of MSC with GFP in 1-well setting, a) without magnet b) with magnet placed on the left end of the 1-well culture slide. D. The responsiveness of the MSCs labeled with nanoparticles were assessed through the exposure to SDF-1 by transwell migration assay, the chemotaxis

and magnetic attraction. Migratory efficacy between FluidMAG-CMX and PCS nanoparticles with chemoattractant and magnetic field application.

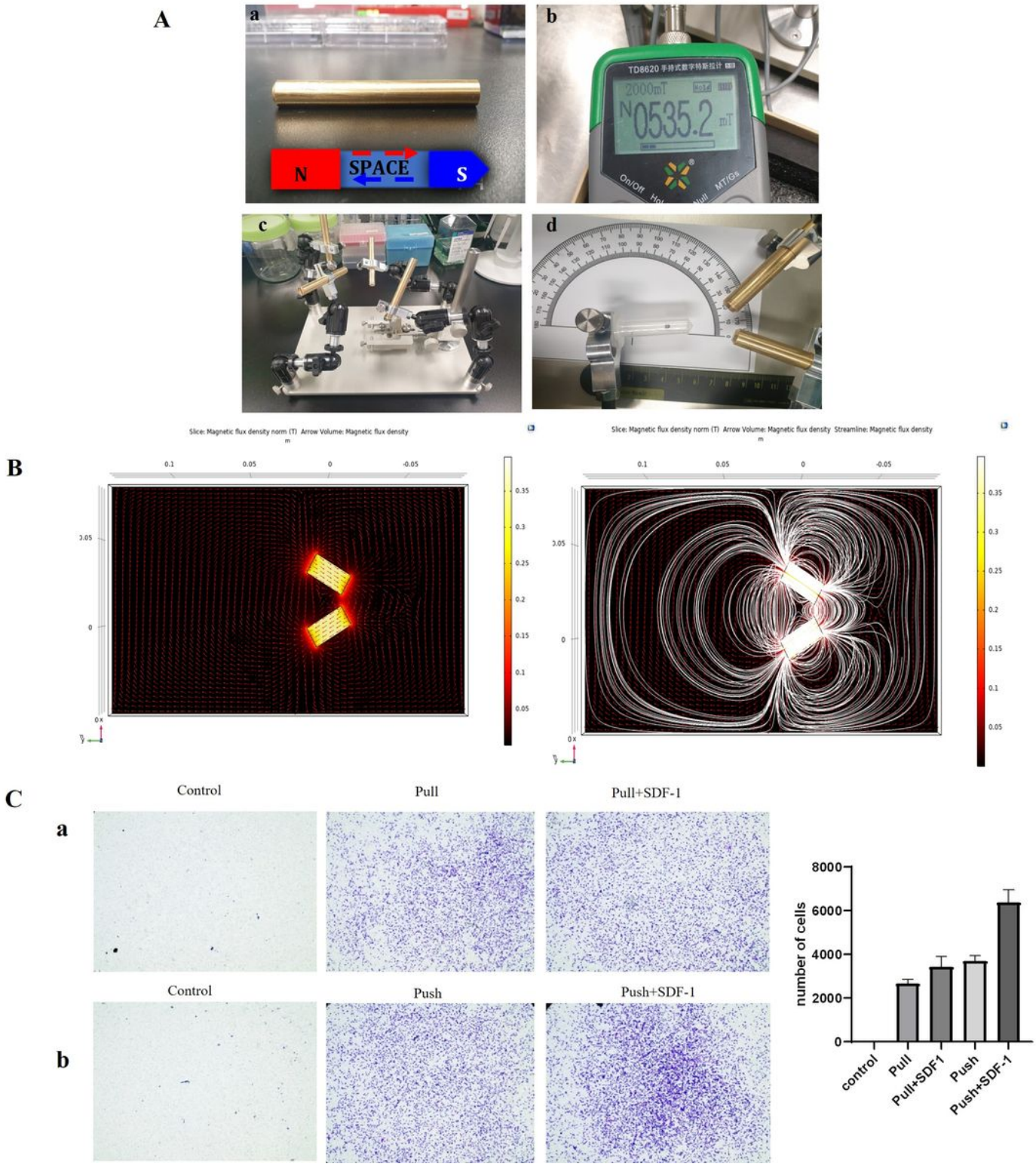

Figure 5

Designing Customized Streotaxic Static Magnet for In Vivo Application. A a) cylindrical magnet with blunt tip with magnetic with opposite poles placed inside to generate high magnetic force, b) magnetic flux

density was measured by teslameter, the customized magnet had magnetic force of 535.2mT at the tip of the magnet. C) Stereotaxic arms customized for free range manipulation of static magnet. **d**) demonstration of magnetic push force, the magnetic force was measured at 172mT. B. Magnetic flux density with two reciprocally positioned magnetic poles was simulated using mathlab to predict the distance of zero force area. D. Prototype experiment was performed by in vitro assessment, Transwell migration assay of PCS nanoparticles labeled MSC; a) NdFeB magnet with magnetic force of 370mT was exposed to the cell as pull force and culture media with 10ng of SDF-1 was placed in lower well for 24h. b.) NdFeB magnet with magnetic force of 172mT was exposed to the cell as push force and culture media with 10ng of SDF-1 was placed in lower well for 24h. E) Number of cells migrated in transwell assay, the magnetic push group with chemoattractant

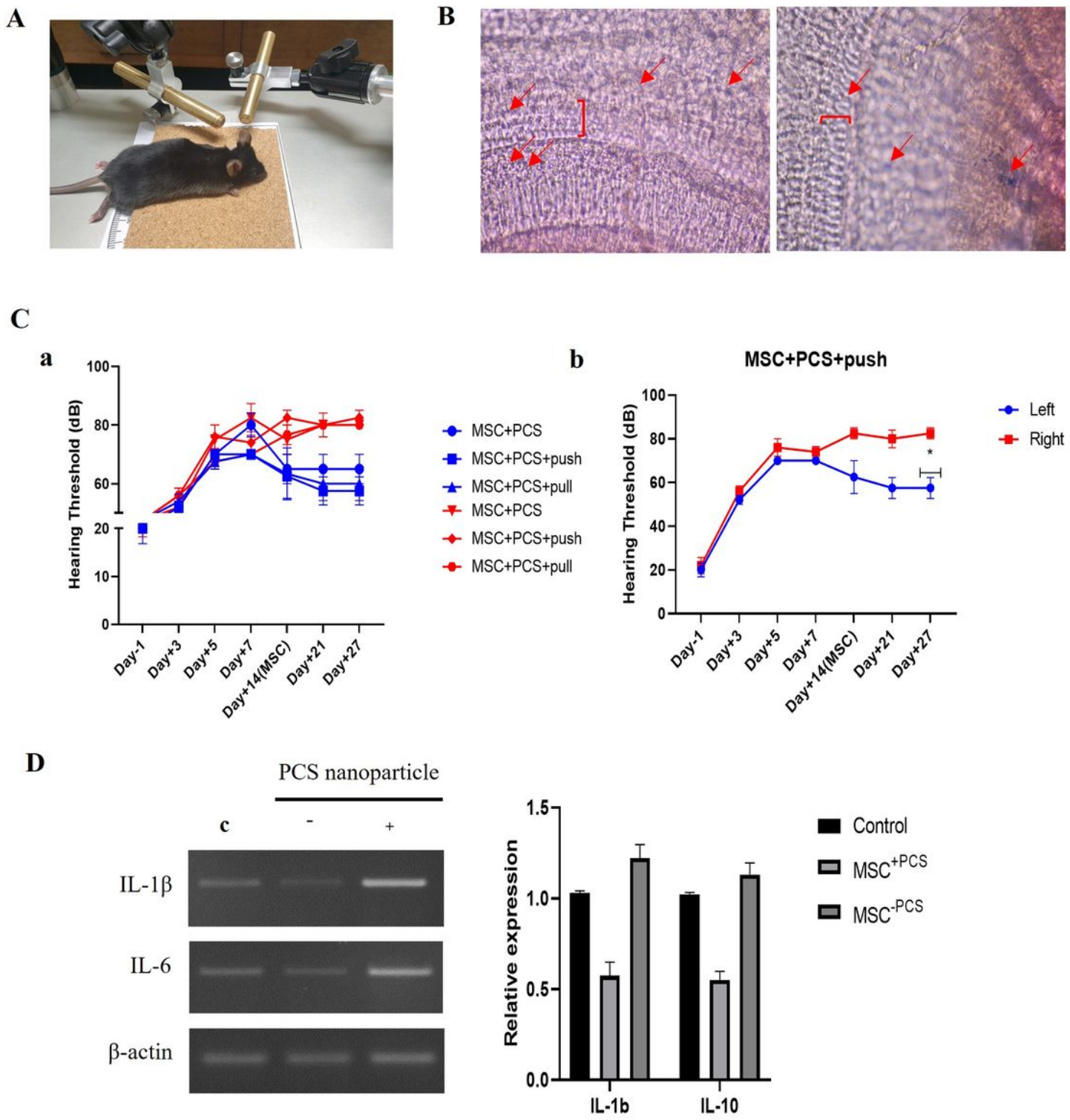

Figure 6

Otoprotective effect of PCS nanoparticle labeled MSCs in Ototoxic animal Model. A. Magnet application in Ototoxic mouse model. B. Bright field imaging of cochlear samples in Prussian blue staining, red arrows indicate iron oxide nanoparticles stained in blue. C. Changes in Hearing Threshold post MSC injection. ABR recordings a) Hearing Threshold measured by ABR 7, 14, 21 days (14, 21, 27 days post ototoxic drug) post MSC transplantation. MSC+PCS group 20% efficacy, group B and C with 40% efficacy.

b) MSC+PCS push group difference between left MSC treated ear vs. right control ear **p < 0.05 vs. control right ear. Data were analyzed by t-test. D. RT-PCR results of pro-inflammatory cytokines in Ototoxic mouse cochlea

Supplementary Files

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