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Magnetic Field-Guided Cell Delivery with Nanoparticle-Loaded Human Corneal Endothelial Cells

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Abstract

To improve the delivery and integration of cell therapy using magnetic cell guidance for replacement of corneal endothelium, here we assess magnetic nanoparticles' (MNPs) effects on human corneal endothelial cells (HCECs) *in vitro*. Biocompatible, 50 nm superparamagnetic nanoparticles endocytosed by cultured HCECs induced no short- or long-term change in viability or identity. Assessment of guidance of the magnetic HCECs in the presence of different magnet shapes and field strengths showed a 2.4-fold increase in delivered cell density compared to gravity alone. After cell delivery, HCECs formed a functional monolayer, with no difference in tight junction formation between MNP-loaded and control HCECs. These data suggest that nanoparticle-mediated magnetic cell delivery may increase the efficiency of cell delivery without compromising HCEC survival, identity or function. Future studies may assess the safety and efficacy of this therapeutic modality *in vivo*.

Keywords

magnetic nanoparticles; human corneal endothelial cells; corneal edema; cell therapy

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Background

Corneal endothelial dystrophies are a significant cause of blindness. When the number of endothelial cells reaches a critical minimum, the endothelium can no longer maintain its function in regulating corneal hydration, and the corneal stroma swells with edema, which leads to epithelial layer sloughing and pain, along with loss of vision.^{1, 2} Current treatments include incisional surgeries such as penetrating keratoplasty (full corneal transplant) or endothelial keratoplasty, transplanting only the inner cornea.^{3, 4} These procedures can be limited by post-operative recovery time, prolonged visual rehabilitation, unpredictable refractive changes, endothelial cell survival, and the number of donor corneas available, particularly in the developing world.^{5–8}

One strategy to address this limitation and increase the number of patients that can be treated with each tissue is to grow human corneal endothelial cells (HCECs) *in vitro*, expanding their numbers, and then deliver them onto the posterior corneal surface.^{1, 9–11} Millions of human corneal endothelial cells (HCECs) can be expanded *in vitro* from a single donor cornea,^{1, 12–14} and they can be injected into the anterior chamber to repopulate the diseased endothelium.^{10, 15} Here we extend this premise by evaluating a potentially biocompatible method for delivering and targeting HCECs with high efficiency, asking whether superparamagnetic nanoparticles (MNPs) could be used to facilitate delivery of HCECs in the presence of a magnetic field without changing their morphology, identity or functional properties.

Methods

Cell culture

Cadaveric donor corneas, preserved at 4°C in Optisol-GS (Baush & Lomb, Rochester, NY), were obtained from the Lions Eye Institute for Transplant and Research (Tampa, FL), the Florida Lions Eye Bank (Miami, FL) and the National Disease Research Interchange (NDRI, Philadelphia, PA). Primary cultures of HCECs were purified and expanded following the method described by Zhu and Joyce (2004) with some modifications. In brief, corneas were rinsed 3 times in M199 with gentamicin 50 µg/µl (Gibco-Invitrogen, Carlsbad, CA). Endothelium pieces attached to Descemet's membrane were carefully stripped off with forceps under a dissection microscope and incubated in growth medium containing OptiMEM-I (Gibco BRL-Life Technologies, Rockville, MD), 8% FBS (Thermoscientific-Hyclone, Logan, UT), 5 ng/mL EGF, 20 ng/mL NGF, 100 µg/mL bovine pituitary extract (Biomedical Technologies, Stoughton, MA), 20 µg/mL ascorbic acid (Sigma, St. Louis, MI), 200 mg/L calcium chloride (Invitrogen-Gibco, Carlsbad, CA), 0.08% chondroitin sulfate (Sigma, St. Louis, MI), 50 µg/mL gentamicin, and antibiotic/antimycotic solution diluted 1:100 (Invitrogen-Gibco, Carlsbad, CA) overnight at 37°C and 5% CO₂ for stabilization. The next day, the tissue was centrifuged at 931 RCF for 6 minutes, washed in HBSS (Gibco BRL-Life Technologies, Rockville, MD) and incubated in 0.02% EDTA (Sigma, St. Louis, MI) for 1 hour at 37°C. Cells were released by mechanical disruption by passing the tissue 15–20 times through a glass pipette. Cells were pelleted and resuspended in growth medium. Isolated cells and pieces of Descemet's membrane from a single cornea were plated in one

well of 6-well or 12-well tissue culture plates pre-coated with FNC Coating Mix (US Biological, Salem, MA) for 15 minutes at room temperature. All cultures were incubated at 37°C in a 5% CO₂, humidified atmosphere. Media was changed every other day. Cell passaging was performed after cultures reached confluency, using trypsin to split the culture in a 1:2 to 1:3 ratio.

Addition of MNPs to HCECs in Culture

Rat anti-mouse IgG1 superparamagnetic MACS MicroBeads (150 µL, 50 nm diameter; Miltenyi Biotec) were centrifuged at 6010 RCF for 10 minutes at 4°C, the supernatant was removed, and MNPs were washed with 500 µL of 0.02% sterile filtered BSA in D-PBS. This was centrifuged again at 6010 RCF for 10 minutes at 4°C, the supernatant removed, and the nanoparticles resuspended in 150 µL of 0.02% BSA in D-PBS. This was placed in a bath sonicator (Fisher Scientific FS 15, Pittsburgh, PA) for 4 minutes at room temperature. The desired volume of MNPs (e.g. 1, 3, 10, 100, or 1000 µL) was then gently delivered, in a spiral motion, to a single well of HCECs that had reached confluence in a 6-well plate (700,000 to 1,200,000 HCECs); the dish was then lightly shaken. HCECs were incubated with the MNPs for 24 hours at 37°C in adherent culture, except for time-dependence experiments where they were incubated for varying time intervals, as noted. Magnetic-HCECs were harvested with 0.05% trypsin (Invitrogen-Gibco, Carlsbad, CA) incubated for 5 minutes at 37°C.

Immunostaining

For immunostaining of the tight junctions of MNP-loaded HCECs, 50,000 cells on passage 3 were seeded on FNC-coated glass coverslips (Carolina Biological Supply Co., Burlington, NC). MNPs were added as described above either at the time of plating or 4 days later, and all cells were harvested after 5DIV. Thus, HCECs were fixed after an overnight or 5-day incubation with MNPs, in 3% paraformaldehyde in PBS for 20 minutes at room temperature, rinsed three times with PBS and permeabilized with 0.05% Triton X-100 in PBS for 3 minutes. After washing twice with PBS and next with 5% non-fat dry milk in PBS, primary antibody (rabbit anti-ZO-1; Invitrogen, Carlsbad, CA 10 µg/ml) was diluted in 5% milk buffer and incubated for one hour at room temperature. One coverslip was incubated with 5% non-fat dry milk in PBS only, as a negative control. Next, after three washes in 5% milk in PBS, coverslips were incubated in the dark with goat anti-rabbit-Alexa 546-conjugated secondary antibody (1:1000; Life Technologies, Grand Island, NY) in 5% milk in PBS for 30 minutes at room temperature. After two washes in 5% milk in PBS and three washes in PBS, coverslips were mounted in Vectashield with DAPI (Vector Laboratories, Burlingame, CA) and imaged with an upright fluorescent microscope Observer Z1 (Carl Zeiss Microscopy, GmbH, Germany). Four 40× images per coverslip were stitched together using Photoshop (Adobe Systems Inc, San Jose, CA) to offer a far view of the culture.

Quantitative Reverse-Transcriptase PCR

RNA was extracted from HCECs (incubated with or without 10 µL of MNPs as described above) using Trizol Reagent (Ambion, Grand Island, NY) and chloroform per the manufacturer's instructions. Epithelial cells were released from human donor corneas, collected in cold PBS and spun at 100,000g for 5 minutes at 4°C. Stromal cells were

cultured as previously described,¹⁶ harvested after two passages and flash frozen in liquid nitrogen. RNA extraction for both cell types was performed with RNAeasy Mini Kit (Qiagen, Valencia, CA) and all samples were DNase-treated following the manufacturer's protocol.

250 ng RNA per sample of human corneal epithelial, stromal, and endothelial (with and without nanoparticles) RNA were reverse transcribed with qScript (Quanta BioSciences, Gaithersburg, MD) in a 10 µL reaction volume. Initial incubation was performed at 25°C for 5 minutes, and the rest of the reaction followed the manufacturer's standard protocol. cDNA was diluted to 35 µL, and 1 µL per well was used for qPCR in quadruplicate using Power SYBR Green Master Mix (Applied Biosystems, Carlsbad, CA). Primers were used at 0.5 µM final concentration; cycles were 50°C for 2 minutes (once); 95°C for 10 minutes (once); 95°C for 15 seconds and 60°C for 1 minute (40 cycles); and 95°C for 15 seconds and 60°C for 15 seconds (once for dissociation curve). Data was analyzed using the Delta Ct method. Specifically, the cycle thresholds (Cts) for the endogenous control, GAPDH, were subtracted from Cts for each cellular identity marker. The resulting exponential values were converted to linear form using 2^{-Ct} , and fold changes for MNP-loaded cells were derived by normalizing to untreated cells.¹⁷

Primers:

ATP1A1 FW - 5'-CGC ACT GTG ATG GGA AGA A-3'
 ATP1A1 REV - 5'-CAC CCG TGA TGA TGT GGA TAA-3'
 COL4A1 FW - 5'-TTA ACA GCA ACG AAC CCT AGA A-3'
 COL4A1 REV - 5'-ATG AAG CAG GGT GTG TTA GTT-3'
 ENO2 FW - 5'-ACC ACA TCA ACT CCA CCA TC-3'
 ENO2 REV - 5'-GTT CTC AGT CCC ATC CAA CTC-3'
 NCAM FW - 5'-ACC AGA CTG CCA TGG AAT TAG-3'
 NCAM REV - 5'-GCT GAT GTT CCG GGT AGA AG-3'
 ZO1 FW - 5'-GCA GCC ACA ACC AAT TCA TAG-3'
 ZO1 REV - 5'-GAA AGG TAA GGG ACT GGA GAT G-3'
 KRT12 FW - 5'-AGA TGC TTC ACA GAG CGA TTA C-3'
 KRT12 REV - 5'-CAA GAG GAG CTG GGC ATT T-3'
 KERA FW - 5'-CGC ACA ATC AAC TCA CAA AGG-3'
 KERA REV - 5'-GGA TGG GCT GGG ACA TAT TAC-3'
 GAPDH FW - 5'-CAA GAG CAC AAG AGG AGA G-3'
 GAPDH REV - 5'-CTA CAT GGC AAC TGT GAG GAG-3'

Electromagnet

An electromagnet was constructed by machining a 1018 cold-rolled steel rod to the following dimensions: back diameter 8 mm by 17 cm length, middle diameter 1.5 cm by 3 cm length, and front diameter 0.5 cm by 2.5 cm length. The front diameter was then sharpened on a sand belt to allow for a more focal magnetic force application. This was fit tightly into a solenoid which was connected to a variable autotransformer voltage source (output 120 or 140 volts, variable autotransformer 0–100; Staco, Hayward, California). Force was calibrated with 10 μ L of a 1:100 dilution of 100 nm MNPs (Chemicell # 4202-5, Berlin, Germany) in glycerol (4080 CP; Sigma, St Louis, MO). Stoke's law ($F = 6\pi\eta Rv$) was used to calculate the force, using 120 volts delivered to the electromagnet for time-lapse recordings of MNPs ($n=3$) traveling at a distance of 12 mm from the electromagnet tip (the working distance of the electromagnet experiments) recorded with video microscopy (AxioVision; Zeiss, Jena, Germany).

HCEC Movement Experiments

300,000 $\pm$ 100,000 cells in 1 mL of media from the control group (HCECs without nanoparticles) were pipetted into a round, 35 mm glass-bottom cell culture dish (MatTek, Ashland, MA) on the stage of an inverted microscope (Zeiss), with an electromagnet (initially powered off) positioned 12 $\pm$ 2 mm to the right of center and 3 $\pm$ 2 mm above the dish bottom, at an angle of 30 $\pm$ 5°. The dish was modified by opening a small window on one side, to allow the electromagnet tip to be positioned closer to the location where cells would be pipetted. The electromagnet was kept in the off position for 20 seconds as time lapse video was recorded to allow for baseline cell/fluid motion calculations. Then the electromagnet was powered on to 120 volts and the variable autotransformer was turned to the maximum 100, which according to force calibration experiments generated a force of 22.8 pN at that distance. Five minutes of time lapse video were recorded. A demagnetizer was applied to the electromagnet for about 30 seconds, and the electromagnet was allowed to cool off for 15 minutes before the next condition of HCECs was prepared. The experiment was repeated with increasing concentrations of 50 nm MNPs (1, 3, 10, 100, 1000 μ L) in triplicate.

Contact Lens Experiments

Soft hydrogel contact lenses (Base Curve 8.3, Diameter 14 mm, Acuvue 2, Jacksonville, Florida) were prepared for cell attachment. To counter their anti-adherence surface coating, the lenses were chemically cross-linked to attachment proteins as follows: lenses were left overnight in 2-(N-morpholino) ethanesulfonic acid (MES) buffer pH 5.0 in a 12-well plate. The next day MES was aspirated, and 650 μ L of 1 mg/mL 1-ethyl-3-(3-dimethylaminopropyl) carbodiimide (EDC) in MES were added to each well to cover the lenses for 30 minutes. The EDC was aspirated and 500 μ L of 2 mg/mL Sulfo-N-Hydroxysuccinimide (SulfoNHS) were added to each well to cover the lenses for 1 hour. The SulfoNHS was aspirated, and 650 μ L of FNC Coating Mix was added to each well and allowed to cover the lenses for 4 hours. The lenses were then stored in phosphate buffered saline (PBS) solution overnight. Twelve-well plates were prepared by adhering various permanent magnets (see below) to the bottom of the well with silicone gel. Magnets were

allowed to set, and then the prepared contact lenses were adhered face-up, above each magnet, again using silicone gel. A modified Slurpee™ straw (7-Eleven, Dallas, TX) was used to scoop each contact lens from underneath so as to avoid contact with the inner surface. The wells were then gently filled with pre-warmed HCEC growth media. 50,000 HCECs, pre-incubated with or without 10 μ L of 50 nm MNPs, were then gently pipetted from 5 mm above each contact lens, using a modified pipette with ruler markings to ensure consistent delivery distance. The pipette was set in a clamp in order to minimize differences in cell delivery. The dishes were allowed to sit undisturbed for 15 minutes and then transferred to an incubator at 37°C and 5% CO₂ for a 24-hour incubation, after which the contact lenses were fixed in 4% PFA and stained with 1:10,000 DAPI (Invitrogen, Carlsbad, CA) and 1:300 Alexa® 488-conjugated phalloidin (Sigma-Aldrich, St. Louis, MO) in PBS. To determine whether there were differences between the density of HCECs on contact lenses amongst the different experimental conditions, analysis of variance (ANOVA) was performed, followed by a post-hoc least significant difference (LSD) test using SPSS version 20 (IBM Corp., Armonk, NY).

Permanent magnets

A series of magnets was examined with properties as delineated in Table 1.

Measurement of Trans-endothelial Electrical Resistance (TEER)

HCECs were cultured on inserts with 0.4 mm pores in 24-well culture plates (Transwell, Corning Costar, Acton, MA) at a density of 20,000 cells/insert and incubated as described above. TEER was measured using an EVOM volt-ohm meter with STX2 Electrode (World Precision Instrument, Inc., Sarasota, FL) for 1 to 5 weeks after plating. TEER measures the apical and basal plasma membrane resistance and the paracellular resistance and is used as an index of monolayer confluence integrity of tight junctions. To calculate final resistance ($\Omega \cdot \text{cm}^2$), the resistance of blank filters were subtracted from those of filters with cells. Four wells per condition were averaged.

Statistical analyses

Power analyses were performed to generate sample sizes for the various experiments. For example, for the magnet-guided HCEC density experiment, Cohen's effect size $d=11.5$, power=0.8, and $\alpha=0.05$, yielded a minimum sample size of 2 per group, although in all cases a minimum group size of 3 was performed. Analyses including ANOVAs and post-hoc t-tests were performed and are noted throughout the results and figure legends.

Results

Viability and Movement of MNP-Loaded HCECs *In Vitro*

Since magnetic nanoparticles could be potentially used in cell therapy to help direct HCECs to the corneal endothelium, we first asked whether adding increasing volumes of 50 nm diameter superparamagnetic nanoparticles (MNPs) is toxic to HCECs in culture. There was no difference in viability, by Trypan Blue exclusion, between control HCECs and HCECs incubated with 10, 20, 100, or 1000 μ L of MNPs after 1 day *in vitro* (DIV) (Fig. 1A–B). To determine whether treating HCECs with MNPs renders the cells magnetic, we tested a wide

range of MNP doses to find the maximum effect with the lowest dose to minimize the possibility of toxicity. Increasing volumes of MNPs were delivered to a confluent monolayer of HCECs in culture and allowed to incubate for one day. HCECs treated with MNPs moved significantly faster in the presence of a constant 22.8 pN electromagnetic force. The speed increased in a dose-dependent manner, reaching a plateau at high concentrations (100 μ L) (Fig. 2A–B, Supplemental Video 1). Furthermore, there was a significant difference in the percentage of MNP-treated HCECs that underwent a change in trajectory in the presence of the electromagnet compared to untreated HCECs (Fig. 2C). We selected 10 μ L as the MNP treatment volume for all subsequent experiments, as there was no significant difference in the percentage of HCECs pulled by the electromagnet for volumes of 10 μ L or more (10 vs 100 μ L: $p=0.689$, $n=3$; 10 vs 1000 μ L: $p=0.507$, $n=3$; Figure 2C). Thus HCECs can be loaded with MNPs in a non-toxic fashion that allows them to respond to external magnetic fields.

Time Course of MNP Loading and Effect on Response to Magnetic Field Application

Next, we asked whether the association between MNPs and HCECs is dependent on treatment time, comparing the velocity of untreated cells to those treated with MNPs and exposed to a 22.8 pN electromagnetic force at various times after MNP loading. There was a significant difference in velocity between all conditions, and HCECs incubated with MNPs for one day were the fastest (Fig. 3A–B). There was no difference in the percentage of cells with altered trajectory between control cells and those exposed to the electromagnet immediately after treatment ($5.35\pm3.30\%$ versus $5.97\pm7.03\%$, $p=0.919$, $n=3$), but there was a significant increase in the percentage of HCECs attracted to the electromagnet for cells allowed to incubate with MNPs over time (Fig. 3C), with significantly more cells pulled after an incubation of one day compared to one month ($p=0.024$, $n=3$). Taken together, this suggests that there is a critical, minimum incubation time period necessary to allow the MNP-HCEC association to occur and that these MNP-treated HCECs have a decrease in their magnetic properties over longer incubation times.

To further examine this relationship, transmission electron microscopy (TEM) was performed on untreated HCECs and on cells treated with 10 μ L of MNPs and fixed three hours, one day, and one month post-treatment. We observed that MNPs were endocytosed into HCECs by a process involving involution of the cell membrane (compare Figures 4A and 4C). This process began early, as intracellular MNPs were noted at three hours post-treatment (Figure 4B), and continued at least through one day post-treatment (Figure 4B). To determine if the decrease in magnetic field response over time was due to a decrease in the number of intracellular MNPs, we counted the average number of MNPs per cell section at one day and one month post-treatment, and found a significant decrease from one day to one month (Fig. 4D), suggesting that intracellular MNPs are eliminated from the cell over time.

Effect of MNP Loading Time on HCEC Viability, Identity, and Function

We then asked whether MNP treatment affects HCEC viability, identity, or function over time. There was no difference in viability, by Trypan Blue exclusion, between the conditions

at one day, one week, and one month post-treatment (Fig. 5A–B), suggesting that these magnetic nanoparticles are not toxic to HCECs acutely or over longer time periods.

To assess whether treatment with MNPs alters the cellular identity of cultured HCECs, we used quantitative real-time PCR (qPCR) to measure mRNA expression of several genes widely used as HCEC-specific markers.^{14, 18–21} In particular, the following cellular identity markers were measured by qPCR at 1 day and 1 month: ATP1A1, COL4A1, ENO2, NCAM, and ZO1. Markers of HCECs were chosen based on their robust expression in untreated cells and comparatively low expression in isolated human corneal stromal and epithelial tissue. To ascertain that there was no contamination of HCEC cultures with corneal stromal or epithelial cells, we also measured the expression of the well-studied cell type markers KERA and KRT12, respectively.^{22, 23} Furthermore, all HCEC samples expressed negligible levels of KERA and KRT12, corneal stromal and epithelial markers, respectively, at both 1 day and 1 month (data not shown). No significant difference was detected in any endothelial, stromal or epithelial marker between the untreated and MNP-treated cells, after 1DIV (Figure 5C), or after 30DIV (Figure not shown). We also examined ZO-1 expression by immunostaining, as it is a tight junction protein critical for HCEC function, and found no obvious differences in tight junction morphology between controls and HCECs treated with MNPs at one and five DIV (Fig. 5D). This suggests that there is no change in cellular identity of HCECs after addition of MNPs.

To assess whether MNP addition affects the functional ability of HCECs to form tight junctions, transendothelial electrical resistance (TEER) was measured for untreated and treated cells at various time points ranging from 1–35 DIV. Although there were differences in the TEER measurements from one experimental preparation to another, particularly at the early 7-day time point, there was no significant difference between untreated and MNP-loaded cells in any of the experimental preparations (Figure 5E). Taken together, these data suggest that nanoparticles do not affect the ability of HCECs to form cell-cell interactions via tight junctions in order to reach a fully-functional monolayer. In addition, there was a plateau in TEER recordings, which on average occurred at 30 days, suggesting that there is a dynamic time period during which the cells continue to form new tight junctions until they reach a steady state.

Delivery of MNP-Containing HCECs by Applied Magnetic Fields

We then asked whether we could harness the magnetic properties of these MNP-loaded HCECs to improve the targeting of selective cell therapy as compared to gravity alone. HCECs with and without nanoparticles were injected 5 mm above 14-mm diameter, FNC-crosslinked, hydrogel contact lenses, and the lenses were positioned above magnets of various strengths and shapes (Figure 6). The presence of a magnetic field significantly increased the density of MNP-loaded HCECs compared to untreated HCECs on the lens surface for all conditions, except for weakest magnetic field of 0.003 Tesla. This is approximately the strength of a refrigerator magnet, and there was no difference between MNP-loaded and untreated HCECs ($p=0.134$; Fig. 6A–B). No difference in HCEC density was observed between all conditions for control HCECs lacking MNPs ($p=0.311$, $n=3$). There was a positive logarithmic relationship between magnetic field strength and HCEC

density ($density = 33.331 \cdot \ln(\text{magnetic field strength}) + 345.73$, $R^2 = 0.81$; Fig. 6A–B) for MNP-loaded HCECs. In the presence of the strongest magnetic field (0.160 Tesla), 86% of MNP-loaded HCECs were delivered to the contact lens as compared to 36% of untreated HCECs, a 2.4-fold increase in HCEC density. Furthermore, variations in magnet shape and positioning appeared to affect HCEC distribution on the contact lens. Specifically, setting the magnet off to the side of center of the contact lens resulted in cells attaching preferentially in the location of the magnet; in addition, distributing the magnetic field along the outer rim of the contact lens resulted in a similar peripheral localization of the cells (Figures 6C–D). Thus, magnetic HCECs can be poised to move in different directions in a variety of magnetic field distributions, with more effective cell delivery than with gravity alone.

Discussion

Here we find that nanoparticle-endocytosing HCECs show no difference in viability, identity, or barrier function compared to untreated cells. Nanotechnology use in medicine has expanded tremendously over the last two decades. Nanomaterials are currently used clinically to augment the potency of prostate cancer therapy, to enhance imaging modalities, and to improve drug bioavailability, among other applications.^{24–30} Nanoparticle drug delivery systems have been designed to target drugs and genes to specific tissues, to evade the immune system, and to study subcellular biological processes.^{31–36} Recently, magnetic nanoparticles have also been explored to deliver cell therapies throughout the body. For example, magnet-assisted delivery of magnetically labeled mesenchymal stem cells has been used following angioplasty to aid with cell retention to aid in regenerating myocardium, and for helping to repair articular cartilage defects.^{37–40} In the eye, spherical iron powder has been used to magnetically deliver cultured rabbit corneal endothelial cells *in vivo* to a rabbit model of endothelial dysfunction by cryo-injury, with significantly improved corneal thickness compared to non-treated eyes.^{10, 15} HCECs containing magnetite oxide superparamagnetic microspheres were similarly shown to be magnetically attracted to corneal stroma in an *ex vivo* human anterior segment model without significantly increasing intraocular pressure.⁴¹

Here we show that 50 nm diameter magnetic nanoparticles can be used for magnetic field-enhanced cell delivery, with approximately a 2.4-fold increased delivery efficiency *in vitro* to a desired surface, compared to gravity alone. The efficacy of this treatment depends on the number of nanoparticles in each cell, and thus the initial nanoparticle loading concentration, and also the strength of the applied magnetic field. We observed a positive, logarithmic relationship between magnetic field strength and delivered HCEC density, as expected.

We found that treating cultured HCECs with increasing volumes of nanoparticles resulted in an increased velocity of the cells in the presence of a constant electromagnetic force, which is likely due to an increased number of endocytosed nanoparticles. We observed, however, that at a treatment volume of 10 μL of nanoparticles and above, there was no significant difference in the percentage of cells with their trajectory altered by the electromagnet. This likely represents the percentage of cells containing a critical load of nanoparticles that

allowed them to be attracted to the electromagnet with sufficient force to change their path. We also observed a time-dependence for endocytosis of the nanoparticles, with a twenty-four hour incubation period appearing to be optimal. Cells that were allowed to incubate with nanoparticles for one month had significantly fewer intracellular nanoparticles, suggesting that nanoparticles initially endocytosed are then removed from the cell during that time period. This may be due to a net exocytosis, or lysosomal degradation and removal of the nanoparticle contents from the cell, or some other mechanism.⁴² If a similar process occurs after transplant *in vivo*, it may allow the magnetic nanoparticles to be shed from the transplanted cells, exit the eye and be excreted from the body.^{42, 43}

Nanoparticles were found to be non-toxic to HCECs *in vitro* (this study), and were previously demonstrated to be non-toxic to ocular tissues *in vivo*, in contrast to micron-scale magnetic particles.^{44, 45} These data are consistent with others' findings, although other nanoparticles may have systemic toxicities.^{41, 46} For example, silica and titanium dioxide nanoparticles, of 70 and 35 nm diameters, injected intravenously into pregnant mice were found to cross the placenta and cause pregnancy complications.⁴⁷ Functionalization of nanoparticles with polyethylene glycol chains has been found to minimize teratogenic effects below a certain threshold injection dose.⁴⁸ Clinical trials have reported that Rexin-G, a cytotoxic cyclin G1 nanoparticle construct, has no dose-limiting toxicity, nor organ-related toxicity, and has minimal drug-related side effects when delivered intravenously.⁴⁹ Nanoparticle-related toxicity is likely to be lower for intraocular magnetic cell therapy, as it would require exocytosis of the nanoparticles out of the cell, diffusion of the nanoparticles across the trabecular meshwork, through Schlemm's canal, episcleral veins, and the veins of the orbit, and other winding veins to eventually reach systemic circulation. Additional *in vivo* studies will be required to further examine the toxicity and efficacy of nanoparticle-mediated magnetic cell therapy, as delivery of MNP-loaded HCECs may demonstrate a different toxicity profile than the naked MNPs previously tested.^{30, 44}

Delivery of HCECs to a surface was followed by tight-junction formation and conversion into a functioning monolayer barrier, based on TEER data. As this represents one of the major functions served by HCECs *in vivo*, these data suggest that these cells could be delivered to replace missing or damaged corneal endothelium. This premise has been born out in short- and long-term rabbit studies *in vivo* in which rabbit CECs were delivered to rabbit recipient corneas after cryo-injury.^{10, 15} Future *in vivo* studies will be required to further analyze the safety and efficacy of this therapy using human magnetic nanoparticle-loaded CECs.

We also noted a qualitative dependence on the geometry of the applied magnetic field. A field directed offset from center resulted in a preferential distribution of cells in that location. Such an offset might be valuable if considering the delivery of cells to the peripheral cornea, or if using a wider offset, even to the trabecular meshwork. For example, delivery of paramagnetic polystyrene microspheres by magnetic attraction to the iridocorneal angle was found to produce a sustained elevation of intraocular pressure, inducing experimental glaucoma.⁵⁰ In the case of corneal endothelial dysfunction, however, we would hypothesize that a central corneal delivery of cells is more likely to prove effective. A limitation of our study is the number of trials of MNP-loaded HCEC magnetic

field guidance (N=3), and future experiments could shed additional light on the biomechanics and physics required to guide these cells to the desired target.

Taken together, these data support the premise that MNP-loaded HCECs may be a plausible modality for treatment of endothelial cell dysfunction in diseases like Fuchs dystrophy and pseudophakic bullous keratopathy. Whether using primary HCECs or stem cell-derived HCEC-like cells,⁵¹ optimizing cell delivery without depending on a surgical platform remains an attractive approach.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

Magnetic nanoparticles (MNPs) do not affect morphology or viability of human corneal endothelial cells (HCECs) in vitro. **(A)** Light microscope image of control and nanoparticle-treated HCECs at 1 day, showing no difference in morphology. **(B)** Viability of HCECs exposed to increasing volumes of MNPs volumes to 1000 μL (non-significant $p = 0.226$, $N=3$).

Figure 2.

HCECs treated with increasing volumes of MNPs moved significantly faster in the presence of a constant electromagnetic force. **(A)** Average velocity of HCECs after MNP loading increased with increasing MNP volumes added to HCECs for 24 hours in vitro (* $p < 0.001$, $N=3$). **(B)** Cumulative percent of HCECs at each velocity demonstrated a rightward shift to higher speeds with increasing MNP application. **(C)** A significantly greater percentage of MNP-treated HCECs underwent a change in trajectory at the time of powering on the electromagnet compared to controls (* $p < 0.001$, $N=3$); there was no difference among treatment volumes $\geq 10 \mu\text{L}$ of MNPs ($p=0.507$, $N=3$).

Figure 3.

Time-dependent association of HCECs and MNPs. **(A)** Average velocity of HCECs after MNP loading increased with increasing MNP exposure time, with an increased noted immediately upon MNP exposure (* $p < 0.01$, $N = 3$) and a further increase by 1 day (** $p < 0.001$, $N = 3$). Cells incubated with MNPs for 1 day incubation moved the fastest in the presence a constant electromagnetic force. **(B)** Cumulative percent of HCECs at each velocity demonstrated a rightward shift to higher speeds with increasing MNP application. **(C)** There was no difference in the percentage of cells with their trajectory altered by the electromagnet powered to on when comparing untreated HCECs with those treated with MNPs and immediately exposed to the electromagnet ($p = 0.919$, $N = 3$), but significantly more cells were pulled by the electromagnet when allowed to incubate with MNPs for at least 1 day (* $p < 0.001$, $N = 3$), and significantly more cells were pulled by the electromagnet after an incubation time of one day, compared to one month (** $p = 0.024$).

Figure 4.

Transmission electron microscopy (TEM) demonstrated MNP surface binding and endocytosis into HCECs. (A–C) TEM of control, 3-hour and 1-day time points after exposure of HCECs to MNPs, as marked, demonstrating both surface binding and endocytosis with involution of the cell membrane (scale bars=200 nm). (D). Average number of MNPs/cell section after increasing times of MNP co-culture. There was a significant increase at 1 day ($*p<0.001$); by 1 month the MNP quantification remained significantly above control levels although with a significant decrease compared to the number at 1 day ($**p=0.035$, $n=10$ HCECs).

Figure 5.

Evaluation of long-term HCEC identity and function after MNP-loading. **(A)** Light microscope images of control and nanoparticle-treated HCECs at 1 month showed no difference in morphology. **(B)** HCEC viability at one day, one week, and one month after loading with 10 μ L of MNPs showed no toxicity of the MNP exposure ($p=0.831$, $N=3$). **(C)** Expression of ALCAM, ATP1A1, COL4A1, ENO2, NCAM, and ZO1 by qPCR normalized for each gene to control HCECs showed no differences between control-and MNP-loaded cells at one day ($p=0.421$, $N=5$) or one month (graph not shown; $p=0.824$, $N=4$). **(D)** ZO-1 immunohistochemistry revealed no difference in tight junction morphology between controls and HCECs treated with MNPs at one and five DIV. **(E)** Trans-endothelial electrical resistance (TEER) measurements showed no difference between MNP-treated cells and control HCECs within each of 3 paired experiments at any time point ($p=0.728$).

Figure 6.

Effect of magnetic fields on delivery of MNP-loaded HCECs in vitro. **(A)** Fluorescence microscopy showing MNP-loaded and control HCECs with nuclei stained with DAPI (blue) and cytoplasmic actin filaments stained with phalloidin (green) on a contact lens surface, delivered under increasing magnetic field strengths (scale bar=100 μ m). **(B)** Density of MNP-loaded HCECs on each contact lens was significantly greater compared to controls (* p <0.001, N =3), with the exception of the weakest magnetic field of 0.003 Tesla (p =0.134, N =3). There was a positive correlation between magnetic field strength and MNP-loaded HCEC density on the contact lens (R^2 =0.81). **(C–D)** Qualitative analysis of HCEC localization on the contact lenses (photograph in C, schematic representation in D), showed a preferential distribution of MNP-loaded HCECs in regions of magnetic field concentration (scale bar=7 mm). For example, setting the magnet off-center from the contact lens (see arrowheads in 3rd and 4th columns) resulted in cells attaching preferentially off-center, while distributing the magnetic field along the outer rim of the contact lens (see arrowheads in 5th column) resulted in a peripheral localization of HCECs.

Table 1

Characteristics of permanent magnets tested in these experiments.

Magnet Description	Magnet Dimensions	Field Measured at 5 mm (Tesla)
N35 NdFeB Custom-made ^{1,*}	two disks of r=1.3589	0.003
N35 NdFeB Disk ¹	r= 3.175 mm, h= 3.175 mm	0.012
N40 NdFeB Arc ²	l=8.25 mm, d=0.75 mm (r _{out} =8.75, r _{in} =8), 140° arc	0.016
N35 NdFeB Ring ¹	r _{out} =6.35 mm, r _{in} =4.7625 mm, h=6.35 mm	0.020
N40 NdFeB Arc ³	w=11 mm, l=10 mm, d=2 mm (r _{out} =9, r _{in} =7), 140° arc	0.031
N35 NdFeB Disk	r=12.7 mm, h=3 mm	0.062
N35 NdFeB Disk ¹	r=1.3589 mm, h=6.0452 mm	0.078
N50 NdFeB Rectangular Prism ⁴	l=w=2.54 cm, h=1.27	0.119
N50 NdFeB Cube ⁴	l=w=h=2.54 cm	0.160

(¹) Magnet City, Miami, FL.

(²) Newland Magnetics, Ningbo, China.

(³) AIM Magnet, Shen Zhen, China.

(⁴) Applied Magnets, Plano, Texas.

* Custom-made magnet: the base of a precision barrier pipette tip (10, SHARP, Denville, South Plainfield, New Jersey) was cut and two of the r=1.3589 disk magnets were permanently glued to opposite sides of the pipette tip, with opposite poles of the magnets facing each other to promote adherence to the tip.

Theoretical maximum magnetic field (Br_{max}) is 1.17–1.21 T for N35, 1.26–1.30 T for N40, and is 1.40–1.46 T for N50 NdFeB. Nd= Neodymium, Fe=Iron, B=Boron.