Sciatic Nerve Regeneration After Traumatic Injury Using Magnetic Targeted Adipose-derived Mesenchymal Stem Cells

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Abstract
Traumatic peripheral nerve injuries constitute a huge concern to public health. Nerve damage leads to a decrease or even loss of mobility of the innervated area. Adult stem cell therapies have shown some encouraging results and have been identified as promising treatment candidates for nerve regeneration. A major obstacle to that approach is securing a sufficient number of cells at the injured site to produce measurable therapeutic effects. The present work tackles this issue and demonstrates enhanced nerve regeneration ability promoted by magnetic targetted cell therapy in an in vivo Wallerian degeneration model. To this end, adipose-derived mesenchymal stem cells (AdMSC) were loaded with citric acid coated superparamagnetic iron oxide nanoparticles (SPIONs), systemically transplanted and magnetically recruited to the injured sciatic nerve. AdMSC arrival to the injured nerve was significantly increased using magnetic targeting and their beneficial effects surpassed the regenerative properties of the stand-alone cell therapy. AdMSC-SPIONs group showed a partially conserved nerve structure with many intact myelinated axons. Also, a very remarkable restoration in myelin basic protein organization, indicative of remyelination, was observed. This resulted in an improvement in nerve conduction, demonstrating functional recovery. In summary, our results demonstrate that magnetically assisted delivery of AdMSC, using a non-invasive and non-traumatic method, is a highly promising strategy to promote cell recruitment and sciatic nerve regeneration after traumatic injury. Last but not least, our results validate magnetic targeting in vivo exceeding previous reports in less complex models through cell magnetic targeting in vitro and ex vivo.

Keywords: nanomedicine, stem cells, magnetic targeting, sciatic nerve, remyelination.

1. Introduction
Peripheral nervous system (PNS) injuries have a high incidence worldwide, estimated between 13 and 23 cases every 100,000 people per year in developed countries.[1] Despite the
The regenerative capability of the PNS compared to that of the central nervous system, nerve recovery after injury is frequently poor, especially when the lesion occurs far from the target organ or tissue. For instance, the sciatic nerve is the longest in the human body and, after lesion, axons are required to regenerate through long distances at a rate of under 1 mm/day.[2] In these cases, nerve recovery after even a moderate injury can take months or years and fail to restore full morphology and function. For these reasons, the development of new therapies is of great importance. Nowadays, PNS injury treatment is often far from optimal and leads to failure in re-establishing nerve sensitivity and motor function. Available strategies differ based on the type of trauma and injury severity, and interventions encompass nerve grafting, nerve transfer, direct nerve repair, fibrin glue, nerve conduit and novel cell-based therapies, the latter rapidly emerging as a useful tool to reach full tissue regeneration by low invasiveness.[3]

When choosing a cell therapy strategy, both transplanted cells and the target tissue should be carefully considered. For instance, in the treatment of sciatic nerve injuries, autologous Schwann cell (SC) transplantation might be regarded as the most suitable method, but SC isolation can be highly invasive necessitating sacrifice of a healthy nerve and requiring a great deal of time to render an adequate cell number for transplantation. Working with systemically transplanted bone marrow mononuclear cells (BMMC), a heterogeneous fraction including mesenchymal stem cells (MSC), our group demonstrated beneficial effects on sciatic nerve regeneration, together with prevention of hyperalgesia in a Wallerian degeneration (WD) model.[4,5] MSC are adult stem cells, originally described only in bone marrow, which possess self-renewal and multi-lineage differentiation potential. They were popularized in 1991 by Arnold Caplan, who reported their ability to give rise to bone and cartilage.[6] Since then, researchers have isolated MSC from many other tissues such as adipose tissue or dental pulp, among others.[7] MSC are currently considered an attractive source of stem cells for tissue engineering and regenerative medicine. The isolation of MSC, however, may prove as invasive as that of SC in several tissues such as bone marrow, which requires an iliac crest aspiration.
under general anesthesia and often several attempts to obtain an adequate sample. In contrast, since the first report by Zuk et al. in 2001,[8] adipose-derived MSC (AdMSC) have been investigated as a promising tool for regenerative cell therapies. AdMSC can be harvested from adipose tissue through a minimally invasive procedure, render high cell yields, proliferate fast in culture, transdifferentiate into both mesodermal and non-mesodermal cells, particularly neurons and glia [9,10] including SC [11,12] and, most relevant, can be safely transplanted due to low immunogenicity.[13,14]

In parallel, advances in nanotechnology increasingly provide novel approaches to solve public health issues. For instance, magnetic nanoparticles are used for many applications such as imaging probes,[15] magnetic hyperthermia [16,17] and drug delivery.[18] These nanoparticles can be directed in a non-invasive way by an external magnetic field gradient to specific target sites. The high susceptibility of the nanoparticles to external fields has recently led to magnetic targeting (MT) as an emerging therapeutic tool for drug, growth factors and gene delivery.[19] Cargo loading approaches tested so far include liposome, micelle encapsulation, polymer coating and surface functionalization, with promising though unequal results.[20–22] Due to their low cytotoxicity, the most widely investigated type of magnetic carriers are the superparamagnetic iron oxide nanoparticles (SPIONs). SPIONs have been extensively validated for cancer therapies either as carriers of anti-cancer drugs [23] or as hyperthermic agents.[24] Although a few studies have recently yielded insight into their use as drug, gene and growth factor carriers for neuroregeneration [25,26] some studies have also shown their utility as cell labelers for field gradient-assisted cell therapies for spinal cord injury using bone marrow-derived MSC (BMMSC) [27–30]

In addition, iron oxide nanoparticle internalization by SC and in vitro magnetic targeting has been recently proposed as a potential method to promote PNS regeneration,[31] although to the best of our knowledge, no in vivo studies have been reported on magnetically targeted MSC for PNS regeneration.
AdMSC have been used to evaluate regenerative capability in different models of peripheral and central nervous system injuries. In a rat sciatic nerve transection model, implanted AdMSC seeded in a biodegradable nerve conduit showed recovery in nerve conduction and motor function as compared to the autologous nerve graft group. Moreover, transplantation of autologous AdMSC into the injured area of the nerve promoted an increase in the rate of regeneration through a neuroprotective and angiogenic mechanism. However, venous grafts with AdMSC did not yield satisfactory results when matrigel was used as the engraftment conductor. Intrathecally transplanted autologous AdMSC in patients with spinal cord injury showed no severe adverse effects for 8 months and induced a slight neural function improvement. We therefore hypothesize that non-invasive and non-traumatic MT-AdMSC transplantation could constitute an effective strategy for sciatic nerve injury. Furthermore, the anatomical position of the sciatic nerve enables the application of a localized external magnetic field using permanent magnets. The present study aims to evaluate a non-invasive method to stimulate cell arrival at the injury site through MT of systemically transplanted AdMSC loaded with anionic SPIONs (AdMSC-SPIONs) to improve AdMSC regeneration capability upon sciatic nerve lesion with potential clinical application.

2. Materials and Methods

2.1. Animals

Experiments involving animals were performed in male and female adult Wistar rats inbred at our institute animal facilities. Seventy-day-old animals were housed in standard cages in a temperature-controlled room (22 °C ± 2 °C) on a 12 h light-dark cycle. Food and water were provided ad libitum. All experiments with animals followed protocols approved by the Committee for care and use of laboratory animals (CICUAL) at Facultad de Farmacia y Bioquímica, Universidad de Buenos Aires (CICUAL; Res(D) N° 2677-19) and were in accordance with the NIH Guide for the Care and Use of Laboratory Animals.

2.2. SPIONs synthesis and characterization
SPIONs were synthesized by a co-precipitation method of Fe$^{2+}$ and Fe$^{3+}$ salts into magnetite phase followed by citric acid adsorption for achieving electrostatic stability in suspension, preventing aggregation and oxidation, as previously described.[36] Briefly, 2.75 g iron (II) chloride tetrahydrate (FeCl$_2$ · 4H$_2$O, 99%) and 1.01 g iron (III) chloride hexahydrate (FeCl$_3$ · 6H$_2$O, 99%) were dissolved in 100 mL of bidistilled water, mixed in a three-neck flask in a N$_2$ atmosphere, and then heated to the reaction temperature of 60 °C. Then, 3 mL of ammonia solution NH$_4$OH (25% w/w) was added at a rate of 1 drop/sec and left to react for 30 min. Afterwards, 72 mL of ammonia solution was added at the same rate until the solution reached a pH of 10.5 to prevent agglomeration due to surface charge. The black precipitate was separated from the dispersion medium with a permanent magnet, mixed with citric acid aqueous solution (CA, 0.02 g/mL) at pH 5.2 and left to react for 90 min at 60 °C to obtain CA-Fe$_3$O$_4$. Finally, the SPIONs were resuspended in bidistilled water at physiological pH, around 7.4. The surface carboxylate groups provided electrostatic stabilization. The concentration [x] of the as-synthesized colloid was determined through Fe (III) reaction with thiocyanate using a UV-VIS spectroscopy method with a Shimadzu UV-2600 spectrophotometer at a wavelength of 480 nm.[37] The concentration is expressed as iron mass per solvent volume.

Isoelectric titration was performed with Zetasizer (MAL500743) to analyze colloidal stability and determine the isoelectric point, and NanoSight Nanoparticle tracking analysis (NTA) 3.1 was used to obtain SPIONs hydrodynamic size. In both cases, colloids were diluted at 1:1000 in MilliQ water and every experiment was performed by triplicate.

2.3. AdMSC isolation and characterization

AdMSC primary cultures were obtained from 70-day-old rat adipose tissue with modifications of a previously described protocol.[8] Briefly, white adipose tissue was removed, mechanically and then enzymatically digested with a mix of 0.05% type II collagenase (Cat. #LS004202, Worthington, NJ, US) and 0.25 % trypsin (Cat. #27250018, Gibco, MD, US) prepared in Hank’s balanced salt solution without calcium or magnesium (HBSS, Cat. #14185052, Gibco) during
60 min at 37 °C with continuous agitation. Tissue was centrifuged at 550 x g for 10 minutes, filtered through a 100-μm mesh to remove all tissue debris and finally cells were plated in Dulbecco’s Modified Eagle Medium (DMEM, Cat, #12100061, Gibco) supplemented with 10% of fetal bovine serum (FBS, Serendipia Lab, Buenos Aires, AR), 1% glutamine (Sigma, NY, US) and penicillin-streptomycin (50 units/mL-50 μg/mL, respectively) and maintained in standard conditions at 37 °C and 5% CO₂. Upon 85-90% cell confluence, culture flasks were washed with HBSS and the cells were incubated 3-4 min at 37 °C with a solution of 0.25% trypsin in HBSS. Trypsinization was stopped with HBSS-10% FBS, detached cells were centrifuged at 300 x g for 5 min and counted in a Neubauer chamber by the Trypan blue exclusion method. Cells were plated at a density of 5000 cells/cm². For all experiments AdMSC were used in passages 4-5. AdMSC were characterized through the expression of common multipotent markers such as CD105, CD90, CD73 and CD29, and the lack of expression of CD45 and CD11b/c. The experimental procedure used to characterize AdMSC is described in the SI file.

2.4. AdMSC internalization of SPIONs and viability assays

AdMSC were seeded at a density of 10⁵ cells in a 96-well plate in 100 μL complete medium and incubated in standard conditions for 24 h. Subsequently, cells were cultured 2 h more in unsupplemented medium and then incubated for 17 h in DMEM-10% FBS doped with SPIONs to reach concentrations in the range of 100 to 500 μgFe mL⁻¹. Wells were finally washed with warm sterile phosphate buffered saline (PBS) to remove all SPIONs residues.

The viability of AdMSC-SPIONs was assessed by two different experimental procedures: colorimetric MTT (3-[4, 5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide) assays [38] and flow cytometry using the fixable Zombie Yellow™ kit (BioLegend, CA, US). For MTT assays, after SPIONs internalization, the medium was replaced with 100 μL fresh DMEM-10%FBS containing 10 μL MTT reagent (ThermoFisher, MA, USA) and incubated in standard conditions for 3 h. The medium was then removed and 100 μL dimethyl sulfoxide was added.
to all wells and mixed for 15 min at room temperature to completely dissolved formazan crystals. Cell viability was determined by measuring absorption at 595 nm in an Amersham Bio-Sciences Biotrak II plate reader. Results represent the mean ± SD of three independent experiments. For flow cytometry experiments, AdMSC-SPIONs were stained with Zombie Yellow™ (Cat #423103, Biolegend, CA, USA), a non-membrane-permeable amine-reactive fluorescent dye useful to differentiate dying from healthy cells based on membrane integrity. Briefly, cells were detached from flasks, counted in a Neubauer chamber, and treated with 100 μl of Zombie Yellow™ during 20 min at room temperature protected from light, following the manufacturer’s protocol. Cells were washed once with 4 mL of PBS-5% FBS, twice with PBS and finally resuspended in 100 μl of PBS-EDTA 1 mM. As positive control of cell death, AdMSC were heated at 95 °C for 10 min.

2.5. Sciatic nerve crush

For in vivo assays, 70-day-old rats were submitted to a reversible Wallerian degeneration (WD) model induced by sciatic nerve crush to induce axonal degeneration and demyelination. For surgery procedures, animals were deeply anesthetized by intraperitoneally injected ketamine/xylazine (75 mg/kg /10 mg/kg). The right sciatic nerve (Ipsilateral, IL) was exposed and compressed at mid-thigh level for 8 seconds using #5 Dumont tweezers. The left non-lesioned sciatic nerve from each animal (Contralateral, CL) was used as internal control (Figure S1A of the SI file). Animals were euthanized at 7 days post-injury (7 DPI) and experiments were conducted on IL, CL, and nerves from non-lesioned animals (Control). On the basis of the characterization of the experimental model, [39] we chose 7d post injury as it is the point of major demyelination and when macrophage recruitment peaks, which makes it the best moment to evaluate AdMSC effect.

2.6. Cell labeling, transplantation, and magnetic targeting

AdMSC and AdMSC-SPIONs were labeled with CMTMR (CellTracker™ Orange (5-(and-6)-(((4-chloromethyl)benzoyl)amino)tetramethylrhodamine), Cat. #C2927, Thermo Fisher
Scientific, MA, US) to track cells after transplantation. Briefly, isolated cells were resuspended in DMEM with 1 μM of CMTMR and incubated for 30 min at 37 °C and 5% CO₂. Then, were washed three times with PBS and resuspended in 300 μl 0.9% saline solution for transplantation. Immediately after surgery, 300 μl vehicle, 6-7x10⁶ AdMSC or AdMSC-SPIONs re-dispersed in saline solution were transplanted through the lateral tail vein. In the AdMSC-SPIONs-transplanted group, a permanent 13 x 23 x 1 mm Neodymium-Iron-Boron (NdFeB) magnet grade N52 with a surface field of about 0.16 T was placed external to the injured sciatic nerve leg for almost 24 h (Figure S1A and Figure S2 of the SI file).

After 7 DPI, animals were reanesthetized for nerve conduction experiments and then sacrificed, IL and CL nerves dissected out and processed for further experiments.

Thus, the experimental design included four groups with five animals per group: Control (non-lesioned), non-transplanted (N/T), AdMSC-transplanted (AdMSC) and AdMSC-SPIONs-transplanted and submitted to a magnetic field (AdMSC/MT). For each experimental procedure 5 animals per group (n=5) were used.

2.7. DC magnetometry

Sample magnetic moment was obtained as a function of the field (H) at room temperature using a Lake Shore 7404 magnetometer operated with maximum fields μ₀Hₘₐₓ = 1.8 T.

For SPIONs magnetic characterization, measurements were performed on 50 μL of the colloidal suspension sealed in heat shrinkable sleeves and sample magnetic moment was divided by nanoparticle mass to obtain specific magnetization.

For uptake determination using vibrating sample magnetometry (VSM), up to 1x10⁶ cells (enough to satisfy VSM sensitivity) were detached from 75 cm² flasks and fixed with 4% paraformaldehyde (PFA) for 20 min at room temperature. Cells were then washed 3 times with PBS for 10 min and finally resuspended in 50 μl PBS, sealed in heat shrinkable sleeves and maintained at 4 °C. Measurements of cell culture magnetization (M) vs. applied field (H) were
obtained at 300 K. From the fit of M vs. H curves the magnetic moment at saturation ($\mu_c$) of the cell culture was obtained. Then, AdMSC-SPIONs uptake was determined as:

$$Uptake = \frac{\mu_c}{M_N N_c}$$

where $N_c$ is the counted cell number using a Neubauer chamber and $M_N$ is the saturation magnetization of the internalized SPIONs.

For VSM analysis, dissected nerves were weighed and sealed in heat shrinkable sleeves. The % AdMSC-SPIONs at the injury site at 7 DPI was estimated from VSM measure of the nerve as

$$AdMSC - SPIONs = \frac{\mu_{nerve}}{M_N Uptake}$$

where $\mu_{nerve}$ is the magnetic moment at saturation of the nerve.

**2.8. Sample preparation for transmission electron microscopy**

For SPIONs characterization, samples were prepared by dropping the colloid on a carbon-coated copper grid (ultrathin carbon/holed carbon, 400 mesh copper grid).

Meanwhile, for SPIONs internalization experiments, AdMSC were incubated with SPIONs as previously described. Briefly, cells were fixed 2 h at 4 °C in 2% glutaraldehyde. After fixation, cells were transferred to 2 mL tubes and centrifuged 5 min at 300 x g, supernatants were discarded, and cell pellets were washed 30 min at 4 °C for three times with 0.2 M phosphate buffer (PB) pH 7.4. Cells were then centrifuged at 550 xg for 10 min until a compact pellet was obtained, and then post-fixed with 1% osmium tetroxide, 2 h at 4 °C. Following post-fixation, samples were washed with MilliQ water, then gradually dehydrated in growing concentrations of ethanol and acetone, and finally embedded in epoxy resin for 36 h at 35, 45 and 60 °C for polymerization. For *in vivo* experiments, sciatic nerves excised from 7 DPI animals were cut in 3x1 mm pieces and then processed as described for AdMSC-SPIONs samples. Semithin (0.5 µm) and ultrathin sections (60 nm) were cut using an ultramicrotome (Leica EM UC7). Semithin tissue sections were mounted onto glass slides and dyed with 0.5% Toluidine blue in sodium carbonate 2.5% (w/v).
2.9. Epifluorescence, confocal and transmission electron microscopy

Cell arrival at the injured nerve was assessed 7 DPI using an Olympus BX100 epifluorescence microscope and confirmed through images obtained with an Olympus FV1000 confocal microscope.

For SPIONs size and structural characterization, transmission electron microscopy (TEM) micrographs and electron diffraction (ED) images were obtained with a JEOL JEM 1210 electron microscope operating at 120 kV. For AdMSC-SPIONs and sciatic nerve analyses, images were obtained with JEOL JEM1400 (120 kV) and TEM-Talos F200C (200 kV) electron microscopes and using low dose and a nitrogen sample cryo-holder.

2.10. Immunofluorescence

For immunofluorescence (IF), IL and CL sciatic nerves were dissected and fixed overnight in 4% PFA-4% sucrose in PBS at 4 °C. Then the nerves were rinsed with 15 % sucrose-PBS and later 30% sucrose-PBS both overnight at 4 °C and stored at 4 °C until processed. Fixed nerves were frozen and cut in 16-μm-thick longitudinal sections in a Microm cryostat (Zeiss, Oberkochen, Germany). Tissue sections were mounted onto gelatin-pre-coated glass slides and allowed to dry for at least 24 h. Sections were rinsed twice with PBS and then three times during 10 min in washing solution PBS-0.1% Triton X-100. Sections were then incubated in blocking solution (PBS-0.1% Triton X-100-5% FBS) at least 2 h at room temperature. Slides were incubated overnight with myelin basic protein (MBP) (Cat. #808402, Biolegend, CA, USA) or tubulin beta 3 (βIII-tubulin) (Cat. #802001, Biolegend CA, USA) primary antibody diluted 1:1000 in washing solution at 4 °C in a humid chamber. Sections were then rinsed with washing solution three times for 10 min and incubated 2 h at room temperature protected from light with goat anti-mouse Alexa fluor 488-conjugated secondary antibody (Cat. #A21121, ThermoFisher) or with goat anti-rabbit Alexa fluor 546-conjugated secondary antibody #A10040, Thermo Fisher) plus 2 µg/mL 4’,6-Diamidino-2-phenylindole dihydrochloride (DAPI, Cat. D9542, Sigma, NY, US) diluted in washing solution for nuclei staining.
Tissue samples were rinsed three times with washing solution and twice with PBS and mounted with Mowiol anti-fading solution for epifluorescence and confocal microscopy analysis. In all cases, controls were done by incubating the samples without the primary antibody.[40]

2.11. Electrophysiological measurements

Electrophysiological analyses were performed as previously described.[39] Briefly, at 7 DPI, animals were anesthetized, and body temperature maintained at 37 °C. For nerve conduction measurements, the distal latency (DL) and the amplitude of the compound muscle action potential (CMAP) were recorded with a portable electromyography instrument (Cadwell Wedge Sierra II, Cadwell Labs, Inc., WA, USA). Electrodes were placed in the soleus muscle and the tail (recording and ground electrodes, respectively). The IL nerve was exposed, as described previously, and the distal areas were electrically stimulated with an intensity of 30 mA. Control nerves were also studied following the same experimental procedure described for IL nerves.

2.12. Image analysis

For the analysis of both longitudinal and cross sections, images of the distal stump were obtained approximately at a 5-mm distance from the crush/injury site.

Quantitative analysis of cell recruitment to the injured nerve, semithin section images and IF preparations was performed using Image J software (Media Cybernetics Inc., MD, USA) on images obtained using an Olympus BX100 epifluorescence microscope.

Cell recruitment to the injured nerve was analyzed by counting CMTMR+ cells and total nuclei in confocal images. At least five images and 10 fields from each image of the crush and distal stumps of the injured nerve from three independent experiments were used for quantification assays.

For nerve semithin section analysis, ten randomly selected fields (0.01 mm2) in ten images obtained from all the experimental groups were used to count intact axons, based on axon shape and myelin morphology.
For IF integrated optical density (IOD) analysis, ten randomly selected fields (0.01 mm²) in five images obtained from each control nerve, and crush and distal stumps from each IL nerve were used to determine MBP and βIII-tubulin IOD. This parameter (derived from optical density or absorbance) is used to indirectly measure the quantity of the positive material contained in a particular region of the sample. [41] It represents the sum of the values of each pixel in the defined region of interest. Briefly, the acquired fluorescence images are loaded to the software and, using the "Split Channels" command, the RGB image is separated into independent grayscale images. This step is useful to avoid interference of channels other than that of interest (green for MBP and red for βIII-tubulin). The background is subtracted with the “subtract background” command and finally the integrated density and mean gray value are measured. IOD values were expressed in arbitrary units as the mean ± standard deviation (SD). Results obtained from crush and distal stumps from the IL nerve were normalized to control nerves.

2.13 Statistical analysis

Statistical analysis was performed using GraphPad Prism 5 software (GraphPad Software, San Diego, CA, USA). Statistical tests, post tests and significance values are indicated in figure legends.

3. Results

3.1 Magnetic nanoparticle and magnet characterization

As the strategy proposed here uses magnetically labelled AdMSC and a magnet’s field gradient, the outcome of this approach will depend on the properties of these tools. Then, before going further into in vivo experiments, it is worth briefly describing magnetic nanoparticle properties and cell-nanoparticle interactions.

SPIONs were synthesized by a co-precipitation method with Fe²⁺ and Fe³⁺ salts into magnetite phase followed by citric acid adsorption (CA-Fe₃O₄; for details see Materials and Methods 2.2).
Structural and magnetic characterization was carried out to verify that CA-Fe₃O₄-SPIONs display adequate physical properties for MT as well as for biomedical applications. Single-magnetic-domain SPIONs are the best option for MT. The magnetic force (\(\vec{F}_m\)) experienced by this type of particle under the influence of a non-uniform magnetic field \(\vec{H}\) is given by:

\[
\vec{F}_m = \mu_0 V (\vec{M} \cdot \vec{v}) \vec{H}
\]

where \(\mu_0\) is the vacuum permeability, \(V\) the nanoparticle volume and \(M\) its magnetization. Then, the force magnitude depends on nanoparticle size and magnetization value at the applied field, as well as applied field gradient.

Roughly spherical nanoparticles were imaged by TEM (Figure 1A), whereas their mean size and polydispersity were determined from a histogram built by counting and sizing over 300 particles from several TEM images (Figure 1B). Analyses revealed a mean size of 9.1 nm and SD 3.0 nm, which is within the single-magnetic-domain size range of magnetite. The ED pattern (Figure 1A, right panel) was indexed by assuming a cubic spinel structure (crystalline space group Fd3m for magnetite and maghemite phases), which indicates good crystallinity and the absence of other spurious phases.

Nanoparticle tracking analysis (NTA, Figure 1C) showed a mean hydrodynamic size of 60.3 and SD 1.6 nm, which is well below the maximum size of 200 nm acceptable for biomedical applications.[42] Room temperature magnetic characterization (Figure 1D) showed a VSM curve typical for SPIONs. This curve is characterized by a zero-coercive field, which means null magnetization when the external field is removed, a highly desirable magnetic condition for biomedical applications. The magnetization curve was well fitted using a Langevin function convoluted with a log-normal distribution of magnetic moments as previously described,[36] indicating a superparamagnetic behavior for SPIONs. Results indicate a large SPIONs mean magnetic moment \(<\mu>\) of about 16440 µB (consistent value with their size) and saturation
magnetization Ms of 101.7 ± 0.2 A m² kg⁻¹ (emu g⁻¹ Fe⁻¹). These values match the required properties for high field responsiveness for MT.

Z potential, assessed through laser Doppler electrophoretic measurements (Figure 1E), rendered a value of -50 mV at physiological pH consistent with good electrostatic stability in aqueous suspension, and isoelectric point at pH 1.45. These results verified that the SPIONs synthesized by coprecipitation have adequate properties for MT and satisfy the requirements for the intended biomedical applications.

3.2 AdMSC internalization of SPIONs and viability assessments

The internalization of SPIONs by AdMSC was confirmed through VSM and TEM analysis. VSM both show iron content and confirms that the iron belongs to the SPIONs. Details regarding AdMSC characterization can be found in Figure S3 of the SI file.

Figure 2A shows magnetization curves for AdMSC culture incubated without SPIONs or with a SPIONs concentration of 100 μgFe/mLDMEM after 2 h of nutrient deprivation. Cell culture incubated without SPIONs revealed a negative slope straight-line which could be related with the diamagnetic behavior of water and organic compounds of cells and culture medium. AdMSC incubated in a medium containing SPIONs showed a typical superparamagnetic curve consistent with the nanoparticle incorporation. Due to the consecutive culture washing processes, the magnetic response is expected to be produced by the SPIONs within the cells. In this way, an uptake of 5.0 ± 0.2 pgFe/cell was calculated using the uptake equation (described in Materials and Methods 2.7).

TEM images of AdMSC incubated with SPIONs were acquired to confirm their internalization in AdMSC endosomes (Figure 2C). In the figure, red arrows point to groups of SPIONs adhered to the cytoplasmic membrane and/or being engulfed. Also, endosomes loaded with SPIONs are indicated with dotted line circles.

A colorimetric method using MTT was used to analyze AdMSC viability after incubation with SPIONs. AdMSC-SPIONs showed no significant differences in viability as compared to
AdMSC without nanoparticles, regardless of SPIONs concentration in the incubation media (Figure 2B). Flow cytometry experiments confirmed this result in the analysis of Zombie Yellow™ dye incorporation (Figure 2D). Consistently, a noticeable variation in the cellular side scattering signal (SSC) was observed in the SSC histogram, with larger cell complexity correlating with larger uptake. This SSC signal difference is mainly caused by nanoparticles both adsorbed in the membrane and internalized into the cytoplasm.[43]

3.3 In vivo MT experiments

3.3.1 AdMSC and AdMSC-SPIONs recruitment into the injured sciatic nerve

As previously described,[39] nerve-crush-induced WD promotes a demyelination process (For further details of the WD experimental model see supplementary Figure S1 of the SI file). Cell arrival at the lesion area was analyzed through confocal and transmission electron microscopy as well as DC magnetometry on samples obtained from dissected nerves 7 DPI from the four experimental groups. Systemically transplanted AdMSC reached the IL nerve and were recruited exclusively into the crush and distal areas. Results show an increase in cell recruitment in the AdMSC/MT group as compared to the AdMSC group (Figure 3A). As previously described with systemically transplanted BMMC,[39] no recruitment of AdMSC was observed in the CL nerve or the proximal stump (data not shown). Furthermore, magnetometry assays carried out over the whole excised nerves dissected 7 DPI (Figure 3B) confirmed the presence of SPIONs exclusively in the IL nerve of the AdMSC/MT group, as evidenced by the superparamagnetic (Langevin type) signal superimposed to the nerve tissue diamagnetic signal. This diamagnetic behavior was observed in all nerve samples without MT. The combination of both results confirms the arrival of AdMSC/MT only at the IL nerve, and the presence of SPIONs in the nerve 7 DPI. In addition, from the nerve magnetic moment at saturation, an estimate of 3.3 % of transplanted cells reached the injured nerve 7 DPI.

In parallel, TEM analysis demonstrated the arrival of cells compatible with AdMSC exclusively at the distal stump of the injured sciatic nerve in the AdMSC/MT group (Figure 4). Panel A
shows the morphology of myelinated axons in sciatic nerves from control animals, while panel B shows that non-treated animals presented a typical WD morphology, with the presence of degenerated axons and large amounts of myelin and axon debris in the distal stump of a crushed sciatic nerve. Panel C shows a partial recovery in axon morphology in animals from the AdMSC/MT group. Cells consistent with AdMSC and nonrepresentative of normal nerve structures are shown in panel C and as a blow-up in panel D. To confirm this observation, isolated cultured AdMSC are shown in panel E.

To further establish SPIONs localization in AdMSC, cryo-TEM analysis was performed in transversal sections of the distal stump of injured sciatic nerves from the AdMSC/MT group. Figure 5 shows degenerated axons of two independent representative fields where the presence of SPIONs is demonstrated and confirmed by zooming into marked areas. Panel A-I and A-II exhibit SPIONs associated with a degenerated axon at 40000X and 80000X magnification, respectively. Panel B-I and B-II show magnifying images of SPIONs associated with degenerated axons marked with red squares in panel B.

3.3.2 Cell transplantation effects on sciatic nerve regeneration

Cell transplantation effects on nerve regeneration were evaluated in vivo in terms of nerve structure through semithin sections analysis, MBP and βIII-tubulin organization through IF, and functionally through nerve conduction assays.

3.3.2a Morphological aspects: Histological analysis

Histological assessment is one of the most reliable methods to demonstrate the success of peripheral nerve regeneration.[44] Thus, tissue structure and organization 7 DPI were evaluated through histological analysis of semithin sections from control, N/T, AdMSC and AdMSC/MT groups (Figure 6). Since the sciatic nerve is a highly myelinated nerve, the structure of an intact nerve consists mainly of highly myelinated axons (Figure 6A). In contrast, complete degeneration is observed in distal stumps of N/T and AdMSC nerves (Figure 6B and 6C,
respectively), where characteristic nerve structures cannot be distinguished and myelin, axon debris and few irregular axons (only in AdMSC-treated nerves) are observed.

On the other hand, in the AdMSC/MT group (Figure 6D), nerve structure is partially conserved, and a large number of intact myelinated axons are observed. Myelinated axons were quantified by measuring and counting those axons with conserved shape and myelin structure (Figure 6E) in several images as described in the Materials and Methods 2.12. Statistically significant differences were found between AdMSC/MT and N/T (mean difference 7.9%, p< 0.01), between AdMSC/MT and AdMSC (mean difference 7.1%, p< 0.05).

3.3.2b Morphological aspects: Myelin and axonal protein distribution

Myelin regeneration following AdMSC and AdMSC/MT transplantation was assessed through the distribution of MBP, a major myelin protein highly sensitive to WD, and βIII-tubulin, a cytoskeletal protein involved in axon guidance and maintenance, useful to evaluate axonal integrity.

IF analyses revealed MBP clustering and a decrease in MBP levels as a consequence of WD in the crush and distal stump in N/T animals. As early as 7 DPI, AdMSC transplant promoted an improvement in MBP organization in the distal area as compared to N/T animals. Moreover, systemically transplanted AdMSC/MT heightened the improvement in MBP organization promoted by AdMSC (Figure 7A).

These assays confirmed that sciatic nerve compression, alone or combined with AdMSC transplantation or AdMSC/MT, left MBP content unaltered in the CL nerve and proximal stump in all experimental groups, which suggests that AdMSCs or AdMSCs transplant combined with MT has no deleterious effects on axon structure and myelination. Most importantly, in the distal stump of the IL nerve, AdMSC/MT greatly improved remyelination resulting in a mean difference of 41.2% compared to N/T group, 27.2% compared to AdMSC treatment (non-statistically significant) and 3.1% respect to the control group (non-statistically significant). As regards of βIII-tubulin organization, Figure 7B shows protein organization as clusters in the
distal stump of N/T animals. Unlike results shown for MBP, no significant changes were observed in animals from the AdMSC and AdMSC/MT groups.

Quantification analysis of MBP and βIII-tubulin IF (Figure 7C right and left panel, respectively) through integrated optical density (IOD) broadly reflected the results described in the previous paragraph. These IF results were corroborated through western blot (see Figure S4 of the SI file). In summary, AdMSC/MT transplant induced a recovery in MBP levels and its distribution all along the fiber as early as 7 DPI. Crushed animals subjected only to a magnetic field did not show an improvement in MBP organization or recovery (see Figure S5 of the SI file).

3.3.2c Functional aspects

The major challenge after peripheral nerve injury is the correct reinnervation of the target site by axonal regrowth and the recovery of normal nerve conduction. To assess functional recovery after cell transplantation and MT, distal latency (DL) and amplitude of the compound muscle action potential (CMAP) were recorded through electrophysiological assays at 7 DPI.[45] DL is the delay from the start of the stimulus to the initiation of the response and depends on the nerve myelination state. In control nerves, as myelin structure was intact, conduction velocity was fast and the time between the stimulus and the response was short. In animals submitted to WD, DL values increased with the extent of demyelination in the injured nerve. Of note, a statistically significant decrease in DL values (mean difference 19%, p<0.05) was registered in animals subjected to AdMSC/MT treatment as compared to N/T animals (Figure 8A). The amplitude of the CMAP can be correlated to the number of functional axons undergoing action potential. In N/T animals, this amplitude decreased due to the low number of axons responding to the stimulus. In AdMSC/MT-treated animals, a non-statistically significant tendency to amplitude recovery was observed as compared to N/T and AdMSC-treated animals (Figure 8B).
4. Discussion

Nervous system injuries exhibit complex pathophysiology, and current pharmacological and surgical approaches have proven to be only partially effective. For these reasons, and given the need for multiple-target therapy, research in neural regeneration has recently and increasingly shifted toward hybrid approaches i.e., combining biological and nanotechnological strategies. A major obstacle to be tackled in both peripheral and central nervous system regeneration is the successful delivery of biological agents to the lesion site. Particularly, the success of cell transplantation in regenerative medicine depends on several factors including the number of transplanted cells, the administration route and cell retention and engraftment in the lesion site.[46] In this scenario, the strategy put forward in the current work may outpace other approaches in improving cell recruitment into the lesion area and providing a continuous source of trophic factors and cytokines through AdMSC-SPIONs-assisted immune response modulation.

Despite the great advances in regenerative medicine using stem cells and specifically MSCs, due to ethical and safety concerns the application of MSCs in clinical treatments remains controversial. To overcome these problems, the use of the MSC secretome, composed by a wide range of molecules (neurotrophins, cytokines) and microvesicles, has been proposed as a cell-free therapy. It has been postulated that AdMSC microvesicles promote an improvement in nerve regeneration in a sciatic nerve transection model. [47] In contrast, in the central nervous system, the intravenous infusion of AdMSC secretome has been shown to ameliorate neuroinflammation and neurological functioning after traumatic brain injury. [48] As the AdMSC secretome is composed by several molecules, different factors could be involved in AdMSC pro-regenerative effects and no single molecule has been identified as a candidate so far.
The current work demonstrates that MT of AdMSC loaded with anionic magnetite nanoparticles, heightens the chemoattractant signals generated in the lesion, and thus enhances transplanted AdMSC arrival at the lesion site and their pro-regenerative action.

Encouraging results have been previously reported for central nervous system injuries using BMMSC, for example, for brain[25] and olfactory tissue injuries.[49] First, Nishida et al. showed that BMMSC labeled with commercial Feridex SPIONs transplanted via lumbar puncture migrate through the cerebrospinal fluid and aggregate in a great number on the surface of the spinal cord owing to the magnetic force provided by an implanted magnet.[28] In turn, Vaněček et al. demonstrated that the distribution and kinetics of BMMSC labeled with poly-(L)-lysine-iron oxide nanoparticles, transplanted in the spinal cord, are affected after the intrathecal implantation of a permanent slab-shaped magnet.[29] In addition, Sasaki et al. demonstrated that MT of Feridex-loaded BMMSC to the site of spinal cord injury accelerates functional recovery in a rat spinal cord injury model, as determined by hind-limb motor function tests.[27]

It should be emphasized that AdMSC offer several advantages over BMMSC,[50] as they can be processed in a less painful way simply by obtaining adipose tissue from the patient through a minimally invasive liposuction procedure. Besides, cell yields are one to two orders of magnitude higher than BMMSC and duplicate faster, thus requiring shorter culture time.[51] AdMSC display high plasticity, having the ability to differentiate into various cell types, particularly into SC.[52] Furthermore, our current results demonstrate that systemically transplanted AdMSC can spontaneously migrate to the injured sciatic nerve. Research studies using autologous AdMSC are thus deemed necessary, given that several phase I/II studies in patients with multiple sclerosis, amyotrophic lateral sclerosis and ischemic stroke, among others, have demonstrated to be safe procedures, although with some controversial results.[53–55]
Iron oxide nanoparticles can be easily synthesized at the nanoscale range and are naturally biocompatible, as iron has its own recycling metabolic pathways. Anionic coated SPIONs have shown high affinity for the cell membrane and are efficiently internalized in cell endosomes, giving cells magnetic properties and responsiveness to external magnetic fields.[56]

Regarding the potential health concerns of the ferrous particles, it has been reported that, once the nanoparticles are inside cell endosomes, they can follow the natural iron degradation pathway. Mazuel at al., using an in vitro model of MSCs 3D spheroids, showed an increase in the mRNA of the ferritin light chain, a subunit of a protein associated with intracellular iron storage. [57] Also, Volatron et al, demonstrated an efficient remediation of potentially harmful iron ions generated by nanoparticles within ferritin and the relative spatial structuration of NPs with ferritins, which have a crucial effect on their degradation and recycling processes. [58]

Regarding cell viability, MTT and flow cytometry assays demonstrated that the CA-Fe₃O₄ used here did not affect cell viability in the concentration range from 100 to 500 µg Fe/mL DMEM. Consistently, the amount of iron injected systemically in animals through cell transplantation of AdMSC-SPIONs was well within a safe concentration range, of about 30 µg Fe. These results, together with confocal and transmission electron microscopy, suggest that the AdMSC-SPIONs systemically transplanted remain in the lesion area along the survival time evaluated.

It is noteworthy that neither viability nor cell differentiation ability were affected by SPIONs incorporation. Evidence in the literature has demonstrated that Resovist (a magnetic resonance imaging contrast agent approved by the FDA) does not affect the viability of BMMSC differentiated in vitro to neuronal-like cells,[59] and that cell labeling via maghemite SPIONs internalization showed no effect on PC12 cells’ ability to differentiate and extend neurites.[60]

Previous studies of spinal cord injury treatments using intrathecal transplantation of BMMSCs with magnet implanted in paravertebral muscles [28,29] or immobilization of animals in magnetic field devices with daily delivery of pulsed magnetic fields[61,62] showed therapeutic effects of magnetic fields used to aid in cell targeting. In this study, we used a noninvasive
method of cell and magnetic field delivery to try and enhance cell targeting with minimal surgical risk. The location of sciatic nerve makes it susceptible to the influence of an external magnet which, given the characteristics of the magnetic field, minimizes animal discomfort while still being effective in cell recruitment. Indeed, the 0.16 T slab shaped magnet enhanced cell arrival at the lesion site, while neither the SPIONs nor the magnetic field caused inflammation in the sciatic nerve. Altogether, these features render the current procedure both safe and efficient. Moreover, the application of the external magnet did not affect animal mobility and allowed advantageous tracking and spatial control of the cells, in contrast to the other above-mentioned approaches.

In previous studies conducted in an animal brain injury model, a magnetic field was applied over 4 h post-transplant, after which magneto-targeted liposome arrival at the lesion site was 10-fold that of free drug intravenously administered.[21] In contrast, in this work, the magnetic field was applied over 24 h post-transplant and assays were conducted 6 days later, ensuring both higher cell recruitment and retention at the lesion site.

Several challenges related to the translation of these therapeutic strategies to clinical trials remain to be solved, as traumatic injuries are the most clinically relevant type of peripheral nervous system lesions. The current therapeutic strategy demonstrates that systemically transplanted AdMSC-SPIONs may be remotely manipulated in vivo by an external magnet. This procedure avoids intraneural transplant into swollen tissue, which may promote further damage, a relevant and by no means minor point to be taken into consideration when evaluating clinical application.

Our results demonstrate that a single systemic transplantation of AdMSC loaded with anionic SPIONs plus 24-h field gradient application is a feasible and safe strategy, rendering at least a 3.3 % of transplanted SPIONs-cell recruitment into the injured nerve still at 7 DPI. This finding constitutes a great improvement on previous reports where similar cell percentages were found only up to 24 h after single intravenous administration.[63] Also, worth highlighting, this
A valuable therapeutic procedure was achieved using low cost commercially available magnets and SPIONs produced by aqueous co-precipitation, which is among the most inexpensive and provides high particle yields. Furthermore, the SPIONs used in the current study are comparable to those already certified by the FDA for use in humans[64] and may be then regarded as potential candidates for nerve repair therapies. The experimental design was optimal for our purposes and model. However, considering other more severe lesions, we are considering the use of larger nanoparticles, i.e. larger magnetic moment and/or larger field gradients.

5. Conclusions

Our results demonstrate that SPIONs synthesized by a co-precipitation method have desirable characteristics for the intended biomedical application in terms of size distribution, stability in solution at physiological pH and cytotoxicity. After their incubation with SPIONs and intravenous administration, AdMSC are recruited in the lesion area.

We have described a new and potentially useful treatment protocol and provide evidence that the combination of magnetic nanoparticle uptake and local static magnetic field treatment can increase the number of AdMSCs at the injury site and improve recovery over AdMSC treatment alone.

In short, our results demonstrate that magnetic targeting constitutes a promising and valuable tool to foster regeneration after sciatic nerve injury by enhancing AdMSC arrival and permanence at the lesion site, with minimal pain and morbidity for patients.

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**Figure 1.** Magnetic nanoparticle characterization. A) Transmission electron microscopy image (TEM, left) electron diffraction image (right) indexed with magnetite cubic spinel structure. B) Size histogram derived from various TEM image analyses. C) Nanoparticle tracking analysis showing concentration vs. hydrodynamic nanoparticle size. D) Room temperature specific magnetization $M$ vs. applied field $H$; red line indicates the best fitted curve using Langevin function. E) Titration confirming colloidal stability in a wide range of pH.

**Figure 2.** Cell nanoparticle interaction. A) Culture cell magnetic moment $\mu$ vs. applied field $H$ for AdMSC incubated with (red) or without (green) SPIONs. Solid lines represent the best fitted curve using Langevin model for cells incubated with SPIONs and a diamagnetic behavior for cell culture incubated without SPIONs. B) MTT showing a slight increase in viability induced by SPIONs. C) TEM images (left) showing SPIONs inside a cytoplasmic vesicle (dotted line circles) and adhered to the cell membrane (red arrows) and zoom showing low nanoparticle compactness. D) Flow cytometry analysis of AdMSC incubated at increasing concentrations of SPIONs from 0 (control) to 500 $\mu$gFe/mL DMEM. Values are expressed as the mean $\pm$ SD. Statistical analysis was performed through one-way ANOVA followed by Tukey’s post-test; no significant differences were observed with respect to control AdMSC. Upper panel shows representative SSC-A vs. Zombie Yellow™ viability marker; gates in every panel indicate the percentage of viable cells. Lower panel shows the variation in SSC signal with increasing concentrations of SPIONs.

**Figure 3.** Magnetically labeled cell recruitment at the injury site. A) Confocal microscopy analysis of transplanted AdMSC and AdMSC-SPIONs transplanted and submitted to magnetic targeting (AdMSC/MT) arrival at the injury site in longitudinal sections of sciatic nerves. Arrows in the images of MERGE and ZOOM columns indicate transplanted cells dyed with 5-((4-chloromethyl)benzoyl)amino)tetrarmethylrhodamine (CMTMR) fluorophore (red); nuclei stained with DAPI (blue). B) Quantification of cell recruitment to the IL nerve showing significant differences between the AdMSC and AdMSC/MT-treated animal groups. C) VSM analysis of dissected nerves, nerve magnetic moment $\mu$ vs. applied field $H$ for non-transplanted (N/T), AdMSC transplanted and AdMSC-SPIONs transplanted and submitted to magnetic targeting (AdMSC/MT) animals. Inset right image shows SPIONs contribution after diamagnetic signal substraction. Values are expressed as the mean $\pm$ SD. Statistical analysis was performed through Student’s t-test *p<0.05

**Figure 4.** Magnetically labelled cell recruitment at the injury site. Transmission electron microscopy analysis of AdMSC arrival at the injury site in transversal sections of A) uninjured (control), B) non-transplanted (N/T) and C) AdMSC-SPIONs transplanted and submitted to magnetic targeting (AdMSC/MT) sciatic nerves. The inset of panel C is shown as a blow-up in panel D. E) Isolated AdMSC in culture for comparison.

**Figure 5.** SPIONs detection in the distal stump of injured sciatic nerves. Cryo-TEM analysis of SPIONs associated with degenerated axons in two independent transversal sections of the distal stump of injured nerves. Insets of panel A and panel B (15000X, 12000X, respectively) are blown up in the subsequent panels. Panel A-I (40000X), panel A-II (80000X), panel B-I (60000X) and B-II (60000X).

**Figure 6.** Cell therapy and MT effects on nerve structure. Analysis of semithin sections of axonal bundles in A) uninjured control sciatic nerve and distal stump of B) non-transplanted (N/T), C) transplanted with AdMSC and D) AdMSC-SPIONs transplanted and submitted to magnetic targeting (AdMSC/MT) sciatic nerves at 7 DPI ($n = 3$). Arrows indicate intact axons; arrowheads indicate irregular axons; asterisks indicate myelin and axon debris. E) Intact axons quantification and statistical analysis was done through one-way ANOVA followed by Tukey’s post-test *p<0.05 **p<0.01

**Figure 7.** Cell therapy and MT effects on nerve protein distribution in terms of immunoreactivity. Epifluorescence images of dissected sciatic nerves. Immunostaining of A) myelin basic protein, MBP, (green),
DAPI (cell nuclei, blue) and merge for control group. C) αIII-tubulin (red), DAPI (cell nuclei, blue) and merge for control group. For the IL nerve from 2nd to 5th row only the merge results are shown for non-transplanted (N/T), AdMSC-transplanted and AdMSC-SPIONs transplanted and submitted to magnetic targeting (AdMSC/MT) animals. Second row shows images of larger sections including crush and distal stumps of the IL nerve (10 mm) taken at 4x magnification (IL Crush to Distal). All other images were taken at 20x magnification. B, D) IOD quantification for both MBP (left) and αIII-tubulin (right), respectively. Values are expressed as the mean ± SD. Statistical analysis was done through one-way ANOVA followed by Tukey’s post-test *p<0.05 **p<0.01 ***p<0.01. Asterisks (*) indicate comparison between distal stumps for AdMSC or AdMSC/MT vs N/T group. Pound symbols (#) indicate comparison of crush area for AdMSC or AdMSC/MT vs N/T group.

**Figure 8.** Cell therapy and MT effects on injured nerve functionality. Results of electrophysiological measurements: A) Distal latency (DL) and B) amplitude of the compound muscle action potential (CMAP). Statistically significant differences in DL values were observed in transplanted and submitted to magnetic targeting (AdMSC/MT) group respect to non-transplanted N/T animals 7 DPI. Values are expressed as the mean ± SD. Statistical analysis was done through one-way ANOVA followed by Tukey’s post-test. *p<0.05.
Figure 2

(A) Magnetic hysteresis loop of AdMSC-SPIONs and AdMSC. (B) Cell viability assay of cells treated with different concentrations of SPIONs. (C) TEM images showing the distribution of SPIONs in cells. (D) Flow cytometry analysis of cell death at different SPION concentrations.