Research Article

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Regenerative Medicine

Stem cell injection in the hindlimb skeletal muscle enhances neurorepair in mice with spinal cord injury

Aims: To develop a low-risk, little-invasive stem cell-based method to treat acute spinal cord injuries. **Materials & methods:** Adult mice were submitted to an incomplete spinal cord injury, and mesenchymal stem cells injected intramuscularly into both hindlimbs. Behavior tests and MRI of the spinal cord were periodically performed for up to 6 months, along with immunohistochemical analysis. Immunohistochemical and PCR analysis of the muscles were used to detect the grafted cells as well as the soluble factors released. **Results:** The stem cell-treated mice presented significant improvements in their motor skills 5 months after treatment. Spinal cord repair was detected by magnetic resonance and immunohistochemistry. In the hindlimb muscles, the stem cells activated muscle and motor neuron repair mechanisms, due to the secretion of several neurotrophic factors. **Conclusion:** Bone marrow mesenchymal stem cell injection into hindlimb muscles stimulates spinal cord repair in acute spinal cord lesions.

Keywords: bone marrow • intramuscular injection • *in vivo* cell tracking • mesenchymal stem cells • muscle regeneration • neurotrophic factors • spinal cord injury • spinal cord repair

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Spinal cord injury (SCI) is a severe and debilitating clinical condition that affects thousands of victims around the world annually, many of whom become permanently disabled, and severely affects quality of life [1]. The only clinically proven therapy for SCI is using methylprednisolone, a glucocorticoid that controls the inflammatory process locally and has only a marginal effect on the recovery if used early after injury, and has been associated with important side effects [2–5]. Thus, it is necessary to search for new therapeutic approaches to treat SCI patients, as well as expand existing knowledge on the cellular and molecular aspects of the disorder.

SCI can be separated into two phases: an initial acute phase, which eventually becomes chronic [6,7]. While in the acute phase there are limited autoregenerative capabilities, at the chronic stage the damage is irreversible. Thus, many therapeutic strategies consider treating the initial phase, in order to ameliorate the final outcome. There are numerous strategies currently being investigated, one of them being the use of adult stem cells. The use of adult stem cells, such as those found in the bone marrow including both populations (hematopoietic and mesenchymal stem cells), has been extensively studied in animal models, demonstrating their ability to reduce inflammation, promote remyelination, neuronal regeneration, axonal outgrowth and ultimately functional recovery [8–23]. Furthermore, autologous bone marrow mononuclear cells or pre-isolated and expanded mesenchymal stem cells have been used in initial clinical trials, with a certain degree of functional improvement [24–27]. However, the neurological improvements to date have been modest.

The scientific rationale for the use of bone marrow stem cells in SCI is mainly based on the anti-inflammatory, neuroprotective and/or regenerative properties of the grafted cells. In the first case, mesenchymal stem cells are known to be immunosuppressive [28–31].

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This reduces the inflammatory response to the injury and in turn reduces the size of the cavity formed, as well as reducing astrocytic and microglial activation [32].

The neuroprotective and regenerative properties of mesenchymal stem cells mainly derives from their ability to produce and secrete numerous trophic factors, exerting a paracrine effect on the surrounding tissue. For example, the secretion of brain derived neurotrophic factor (BDNF), nerve growth factor (NGF) and vascular endothelial growth factor (VEGF) are known to stimulate neuronal sprouting and axonal outgrowth [33,34]. The expression of other factors such as *ninjurin 1* and 2, *netrin 4*, Neural Cell Adhesion Molecule (NCAM), Robo1 and Robo4 are known to be implicated in neuronal migration and axonal regeneration [33,35]. Furthermore, mesenchymal stem cells are capable of removing potentially damaging debris that appears after a spinal cord lesion, such as matrix metalloproteinases I and MMP2 [36–38]. Thus, mesenchymal stem cell injection is a potentially useful tool for SCI treatment. However, direct, intraspinal stem cell injection poses certain problems. For example, the inflammation and hemorrhage present during the acute phase of a spinal cord lesion may be a dangerous niche for the stem cell engraftment, causing their death before their trophic activity may be in effect. Furthermore, it is very difficult to perform the surgical procedure during this phase, aggravating the injury. Therefore, a less invasive intervention may be necessary to guarantee stem cell survival and subsequent spinal cord regeneration.

One possible method to exert a possible regenerative/repair effect on the damaged spinal cord is to inject bone marrow-derived mesenchymal stem cells into the muscle tissue instead of directly in the spinal cord. This has been performed both by other groups as well as in our lab using amyotrophic lateral sclerosis mouse models [39,40]. In these works, the bone marrow-derived stem cells released neurotrophic factors, such as glial-derived neurotrophic factor (GDNF), which were captured by the axon terminals of the neuromuscular plates and retrogradely transported into the neuronal cell body of the spinal cord. Once in the spinal cord, the soluble factors rescued the degenerating motor neurons by activating antiapoptic proteins and inducing prosurvival mechanisms.

The aim of this current study is to confirm if the same effect observed in the amyotrophic lateral sclerosis models commented on previously can also be extrapolated to spinal cord lesions. To this end, the mesenchymal stem cells will be transplanted into the hindlimb muscles of mice submitted to a thoracic, incomplete lesion, allowing a certain degree of recovery. The results of this work will demonstrate that a simple surgical intervention, such as autologous bone marrow transplantation into skeletal muscle tissue, is capable of enhancing motor neuronal survival, and ultimately the motor function recovery.

Materials & methods

Animal care

All the experiments with animals have been performed in compliance with the Spanish and European Union laws on animal care in experimentation (Council Directive 86/609/EEC), and have been analyzed and approved by the Animal Experimentation Committee of the University Miguel Hernandez and Neuroscience Institute, Alicante, Spain. All efforts were made to minimize suffering. Mice were bred and maintained in our animal facilities. Two- to three-month-old C57/B6 mice were used as experimental mice for the SCI. For the mesenchymal stem cells, the bone marrow of 2- to 3-month-old green fluorescent protein (GFP) transgenic mice were used.

Spinal cord injury

The SCI was induced using a similar approach reported in [41]. Briefly, a laminectomy of the thoracic vertebra segment 9 was performed without damaging the dura-mater. A bilateral clamp, using a laminectomy forceps (Fine Science Tools, Heidelberg, Germany) compressing the sides of the spinal cord but leaving 0.5 mm of space in between, was performed for 30 s on the exposed spinal cord, then removed and skin sutured. Before performing the surgical procedure, 0.1 mg/kg of buprenorphine (Buprex, Schering-Plough, Madrid, Spain) was injected into the mice. Isoflurane (Esteve Veterinary, Milan, Italy), an inhalational anesthesia, was used, and the mice was placed on a stereotaxic apparatus (Stoelting, Wheat Lane Wood Dale, IN, USA). The animal was monitored, and anesthesia concentration was controlled.

For the hindlimb surgical intervention, performed immediately after the spinal cord lesion, the hindlimb muscles were exposed and 10 µl of either culture medium (sham control) or 1 million mesenchymal stem cells (TX-MSC) were injected, in a similar approach as our previous publication [40].

Bone marrow extraction & mesenchymal stem cell culture

Two- to three-month-old GFP-mutant mice were sacrificed by cervical dislocation and the femurs dissected. The procedure used was similar to our previous works [42,43]. Bone marrow was extracted from the femurs and dissociated mechanically to obtain a single-cell suspension. Then, the cells were washed, centrifuged and resuspended in culture medium, which consisted

in D-MEM (Invitrogen) supplemented with 15% FBS (Biochrom AG, Berlin, Germany), and 100 U/ml penicillin/streptomycin (Sigma-Aldrich, St Louis, MO, USA). The cells were then placed in culture flasks to isolate the plastic-adherent cell population. The culture medium was changed twice a week and the cells replated when needed, for a total of 3–4 weeks in culture (passage 4–5) to obtain the necessary cell number.

Behavior tests

Treadmill and footprint tests were performed on a monthly basis similar to our previous work [44]. The tests were performed on a monthly basis for up to 6 months after the surgical intervention. A total of 12 control and 12 experimental mice were used for this test.

The treadmill test consisted of placing the mouse in a lane that pushed the animal to a shock grid (0.4 mA). In this manner, the animal must run to avoid the shock. Each mouse was placed on the treadmill five times, with adequate rests between trials, in order to obtain the average maximum speed.

In the case of the footprint test, the hindlimb paws were painted and the mouse placed on a dark tube with a large dark box in the end. A 60-cm strip of paper was placed under the tube, so that when the animal walked towards the box, its footprints were left on the paper. Stride length was measured as the distance from the tips of the toes of one paw to the following step, and the average of ten distances was calculated per trial.

MRI

For the MRI experiments, mice were anesthetized in an induction chamber with 3–4% isoflurane in medical air and maintained with 1–2% isoflurane during the process. Anesthetized animals were placed in a custom-made animal holder with movable bite and ear bars and positioned fixed on the magnet chair. This allowed precise positioning of the animal with respect to the coil and the magnet and avoided movement artifacts. The body temperature was kept at approximately 37°C using a water blanket and the animals were monitored using a MRI-compatible temperature control unit (MultiSens Signal conditioner, OpSens, Quebec, Canada). Experiments were carried out in a horizontal 7 T scanner with a 30-cm diameter bore (Biospec 70/30v, Bruker Medical, Ettlingen, Germany). The system had a 675 mT/m actively shielded gradient coil (Bruker, BGA 12-S) of 11.4 cm inner diameter. A 1H rat brain receive-only phase array coil with integrated combiner and preamplifier, no tune/no match, in combination with the actively detuned transmit-only resonator (Bruker BioSpin MRI GmbH, Germany) was employed. Data were acquired with a Hewlett-Packard console running Paravision software (Bruker Medical GmbH, Ettlingen, Germany) operating on a Linux platform.

T2-weighted anatomical images to position the animal were collected in the three orthogonal orientations using a rapid acquisition relaxation enhanced sequence (RARE), applying the following parameters: field of view 40 × 40 mm, 15 slices, slice thickness 1 mm, matrix 256 × 256, effective echo time 56 ms, repetition time 2 s, RARE factor of 8, 1 average and a total acquisition time of 1 min 4 s [45–47].

To quantify the rate of spinal cord repair, the average illuminated pixel density of the lesion in each mouse was calculated and normalized to the average illuminated pixel density of an adjacent region of the spinal cord. The average illuminated pixel density was calculated using image processing software (ImageJ, National Institute of Health [NIH], USA). In this manner, a value of 1 would indicate that the pixel density of the lesion and adjacent spinal cord was the same, while below and above 1 would indicate that the lesion was darker or lighter, respectively. The spinal cord lesion in the MRI was observed as a darkened region, thus, the relative value obtained would be below 1.

Immunohistochemistry

The mice were anesthetized with isoflurane and spinal cords fixed with 4% paraformaldehyde in phosphate buffer (pH 7.4) overnight. After fixation, the spinal cords were placed in Osteosoft (Merck Millipore) solution for 5 days, in order to decalcify the vertebrae. Then, the tissue was placed in paraffin and transverse sections of 16 μm were obtained and mounted on slides. Only the sections where the SCI was performed (at the T9 level) were studied.

First, the tissue samples were permeabilized with 0.25% triton, then incubated at room temperature in PBS with 10% goat serum and 5% bovine albumin to block nonspecific binding of the primary antibody. Afterwards, the slides were incubated overnight with one of the following primary antibodies: mouse or rabbit anti-GFP (1:200, Molecular Probes, OR, USA), mouse anti-Tuj1 (1:1000, Covance Madrid, Spain), rat anti-GFAP (1:500, Calbiochem, Merck Millipore, Billerica, MA, USA), rabbit anti-BDNF (1:200, Santa Cruz Biotechnology, CA, USA), and sheep anti-neurotrophin-3 (NT-3) and anti-neurotrophin-4/5 (NT-4/5) (1:100 in both cases, Chemicon/Millipore, MA, USA). The slides were then incubated with one of the following secondary antibodies: anti-mouse Alexa Fluor 488 (1:500, Molecular Probes, Life Technologies, Madrid, Spain) for GFP, and biotinylated secondary antibodies for the rest (1:200, Vector Laboratories, CA, USA), which were then incubated with

streptavidin conjugated with Cy3 (1:500). For nuclei staining DAPI (Molecular Probes, Life Technologies, Madrid, Spain) was used. In samples where DAB staining was used instead of immunofluorescence, secondary antibodies conjugated with peroxidase were used and the tissue counterstained with cresyl violet (Acros Organics, Belgium). The slides were then analyzed using a Leica fluorescence microscope and images taken using the system's image software (Leica DMR, Leica Microsystems, Barcelona, Spain).

For motor neuron counting, a total of 10 sections were used per mouse and group (sham- and stem cell-treated mice, at 2, 4 and 6 months) staining for Tuj1, a motor neuron marker. The average number of motor neurons per section was calculated per group.

Conventional & real-time, quantitative PCR

Total mRNA of the cells was isolated using the Trizol protocol (Invitrogen Life Technologies, Spain). In the case of standard PCR analysis, the mRNA was reverse-transcribed using the Quantitect Reverse Transcription kit (QIAGEN), processed with the QIAGEN Multiplex PCR kit, and run on the QIAxcel apparatus. For real-time PCR, mRNA was reverse-transcribed to cDNA and amplified using Power SYBR Green Master mix (Applied Biosystems Foster City, CA, USA). The samples were analyzed in triplicate using the StepOne Plus Real-Time PCR system (Applied Biosystems, Foster City, CA, USA). Quantification was performed using the ΔC(T) method relative to GAPDH (forward: AGGTCGGTGTGAACGGATTTG; reverse: GGGGTCGTTGATGGCAACA), and normalized with respect to control conditions, as in previous reports [43]. The primer sequences used were taken from the PrimerBank webpage [57], while the muscle markers were taken from a previous publication [48]: NT4 (forward: TGAGCTGGCAGTATGCGAC; reverse: CAGCGCGTCTCGAAGAAGT), BDNF (forward: TCATACTTCGGTTGCATGAAGG; reverse: GTCCGTGGACGTTTACTTCTTT), NT3 (forward: AGTTTGCCGGAAGACTCTCTC; reverse: GGGTGCTCTGGTAATTTTCCTTA), GFP (forward: CTGCTGCCCGACAACCA; reverse: GAACTCCAGCAGGACGACGACCATGTG), Nnt (forward: GGGTCAGTTGTTGTGGATTTAGC; reverse: GCCTTCAGGAGCTTAGTGATGTT), Snx10 (forward: AGAGGAGTTCGTGAGTGTCTG; reverse: CTTTGGAGTCTTTGCCTCAGC), Ankrd1 (forward: GCTGGTAACAGGCAAAAAGAAC; reverse: CCTCTCGCAGTTTCTCGCT), Rtn4 (forward: TGCCTTCATTGTTTGTCGGG; reverse: TTCCTAGCTGCTGATAGGCGA), Mt2 (forward: GCCTGCAAATGCAAACAATGC; reverse: AGCTGCACTTGTCGGAAGC), Myf5 (forward: AAGGCTCCTGTATCCCCTCAC; reverse: TGACCTTCTTCAGGCGTCTAC), Mef2c (forward: ATCCCGATGCAGACGATTCAG; reverse: AACAGCACACAATCTTTGCCT), Myog (forward: CTGTTTAAGACTCACCCTGAGAC; reverse: GGTGCAACCATGCTTCTTCA).

Statistical analysis

Statistical significance between control and experimental groups was calculated with Sigmaplot v.12.0 software (Systat Software, San Jose, CA, USA), using the one-way ANOVA test, establishing the level of significance at $p < 0.05$. Values are measured as mean ± standard deviation.

Results

Stem cell injection into the hindlimb muscles improves motor functions in mice with SCI.

The mice were submitted to the surgical intervention after SCI and separated into two groups: control mice where only culture medium was injected (sham) and the experimental group which was injected with 106 bone marrow-derived mesenchymal stem cells isolated from GFP-transgenic mice (TX MSC). The culture medium or stem cells, depending on the experimental group, were injected into the quadriceps femoris of the mice, with a total volume of 10 µl in each limb. After the treatment, the mice were analyzed on a monthly basis, submitting them to behavior tests and analyzed by MRI. In the case of the behavior tests, two tests were performed, treadmill and footprint. Since the crushing injury that was performed on the mice resulted in an incomplete lesion of the spinal cord, the mice retained at least part of their hindlimb motor skills. In the case of the sham controls, there was no indication of improvement in either of the two behavior tests performed (Figure 1A). The histograms are represented as the relative value with respect to the scores obtained from each mouse in the first month. The scores obtained from each mouse in the behavior tests vary greatly from each other; thus, to study the progress of the mice, each one is studied individually. To this end, the scores obtained by each mouse in the behavior tests are divided by the initial score (obtained in the first month), obtaining a relative value (as shown in Figure 1A), so that if the scores obtained in the consecutive months were greater than 1, this indicated that the mouse presented improvements in the behavior tests compared to the initial value, and vice versa (i.e., a relative value below 1 indicated that the mice worsened).

In the first analysis of the footprint test, performed 1 month after the surgical intervention, the footprints were mostly incomplete, with the mice mainly dragging one or both hindlimbs throughout the test (Figure 1B).

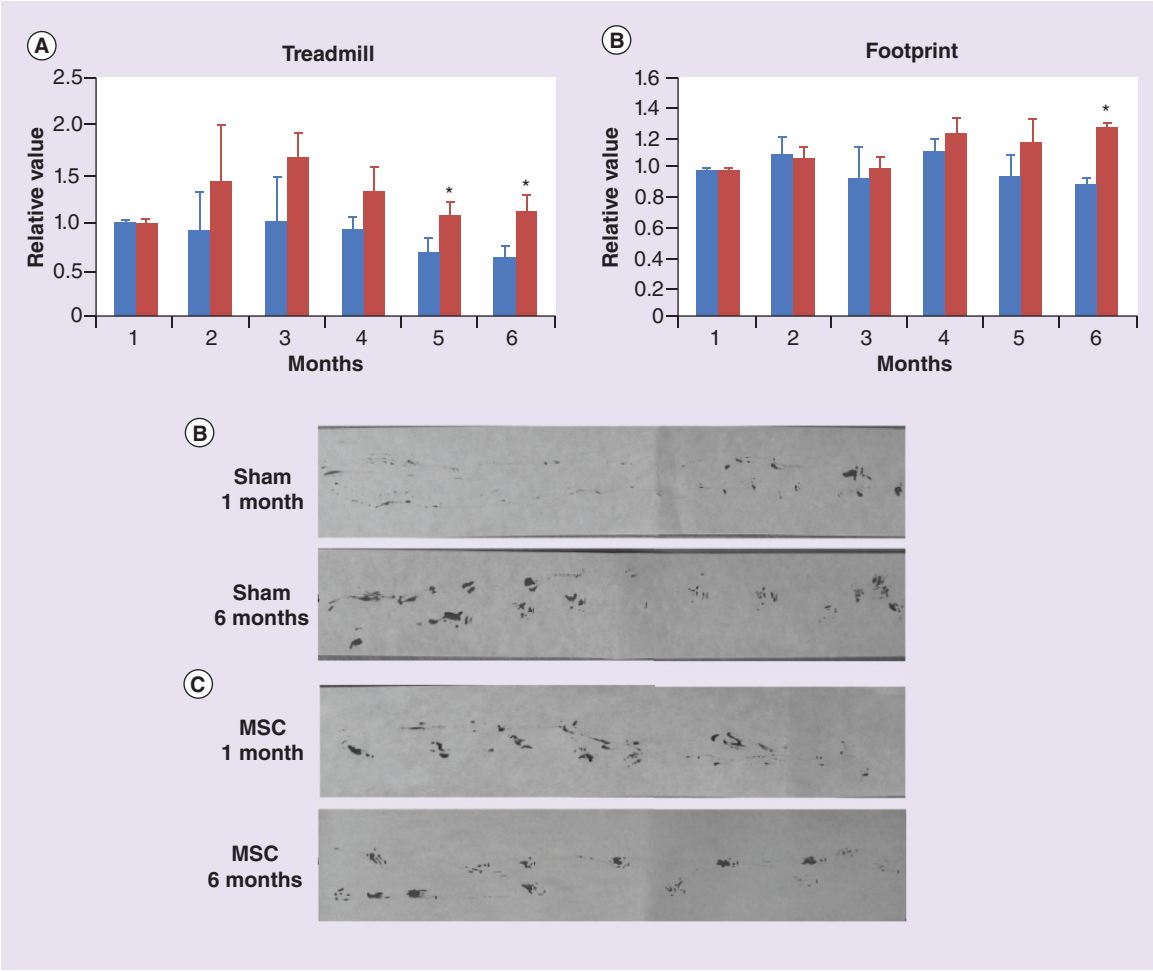

Figure 1. Results of the treadmill and footprint tests. (A) Histograms depicting the results obtained from the treadmill (maximum speed attained) and footprint (stride length) tests. The blue bars represent the sham controls, which were compared with the mesenchymal stem cell-treated group depicted in red (n = 12 in each group). Both groups were compared to the values obtained in the first month analyzed, which was normalized to 1, so that values above and below 1 indicate improvement or loss of motor skills, respectively. **(B & C)** Image taken from a sham control **(B)** and a mesenchymal stem cell-treated mouse **(C)** of the footprint assay at the first and last month of the study. In the first analysis, both groups showed clear loss of proper walking skills, with few complete footprints. At 6 months, the sham control presented a small degree of improvement, while the stem cell-treated mouse presented an almost normal walking ability.
*$p < 0.05$.
MSC: Mesenchymal stem cell.
For color images please see online www.futuremedicine.com/doi/full/10.2217/rme.14.38

However, 6 months after the intervention, the footprints were more pronounced with a larger number of complete footprints, although the stride length remained unchanged.

In the case of the mice treated with mesenchymal stem cells, there was a significant improvement compared with initial values (scores obtained in the first month of analysis) at 5–6 months after the intervention (Figure 1A & C). This was observed both in the treadmill and the footprint tests. In the latter case, at 1 month postintervention the same paw-dragging and incomplete footprints were observed. However, at 6 months, the footprints were almost as those observed in healthy mice, in terms of the full footprint (palm and fingers), with little to no dragging.

Mesenchymal stem cells accelerate spinal cord repair

MRI was used to analyze the progression of the injury in the spinal cord during the whole time of experimentation, on a monthly basis. The injury could be observed as a darkened region in the spinal cord, corresponding to a thinning of the damaged area (Figure 2A). Initially the images observed in both experimental groups were very similar; however, as the months passed, the spinal cords of the

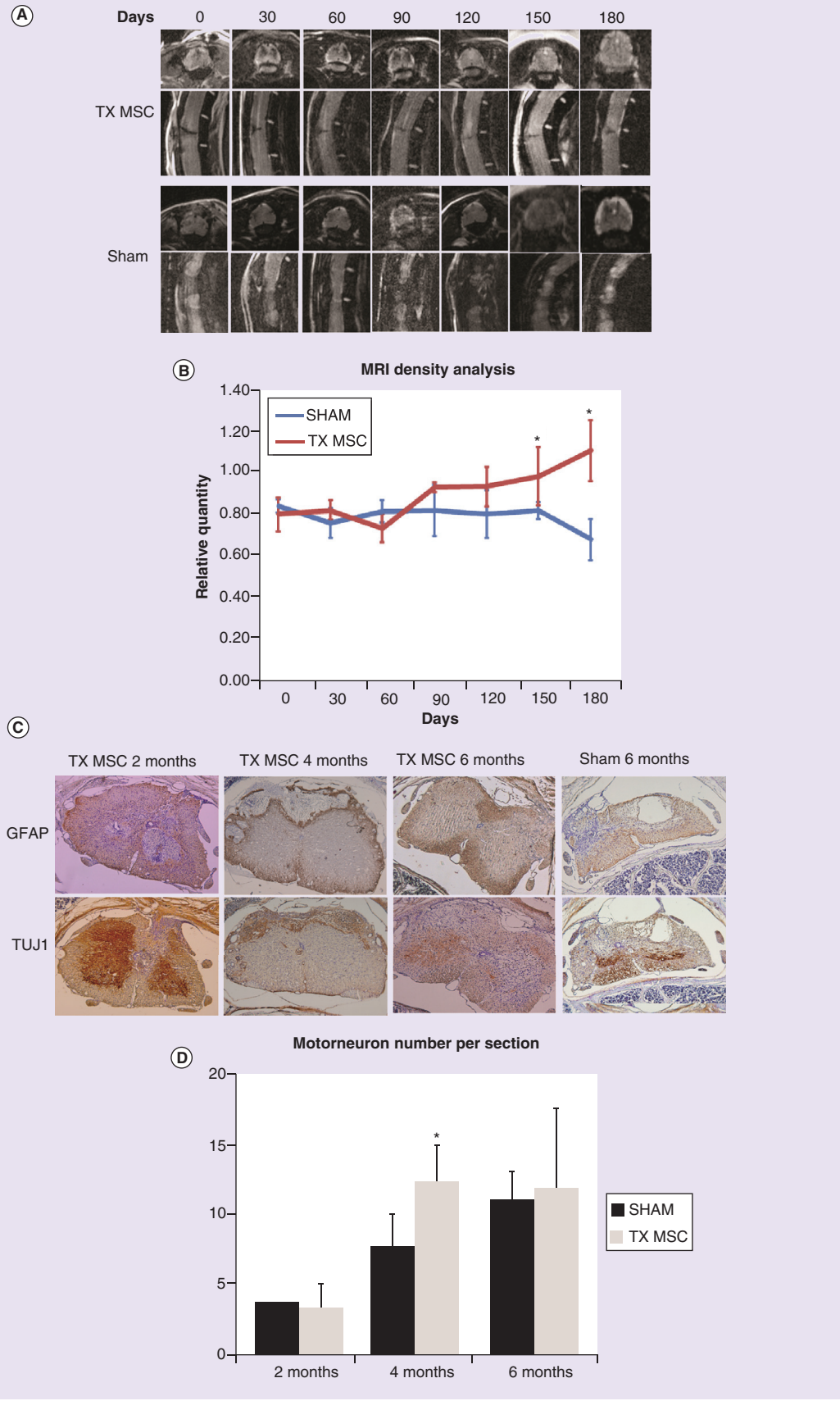
A
Days
0
30
60
90
120
150
180
TX MSC
Sham
B
MRI density analysis
SHAM
TX MSC
Relative quantity
1.40
1.20
1.00
0.80
0.60
0.40
0.20
0.00
0
30
60
90
120
150
180
Days
C
TX MSC 2 months
TX MSC 4 months
TX MSC 6 months
Sham 6 months
GFAP
TUJ1
D
Motorneuron number per section
20
15
10
5
0
2 months
4 months
6 months
SHAM
TX MSC

Figure 2. MRI and histological analysis of the spinal cords (see facing page). (A) MRI images taken from a TX MSC and sham control mice, at different time points up to 6 months after the surgical treatment. The top images of each group correspond to horizontal sections and the bottom images to sagittal sections. **(B)** MRI density analysis of the images taken at the different time points. In the histogram, relative quantity is the average illuminated pixel density of the lesion with respect to nearby healthy spinal cord. n = 12 in each group. **(C)** Immunohistochemical images of TX MSC at 2, 4 and 6 months (sham-injected mice also shown at 6 months) after the spinal cord lesion and stem cell injection, staining for GFAP and Tuj1 and counter-stained with violet-cresyl. Images taken at 100×. **(D)** Histogram depicting the number of motor neurons detected (Tuj1+) per section in the sham and treated groups. n = 4 in each group.
*$p < 0.05$.
TX-MSC: Mesenchymal stem cell treated mice.
For color images please see online www.futuremedicine.com/doi/full/10.2217/rme.14.38

mice treated with mesenchymal stem cells seemed to visually improve, with a progressively smaller darkened/damaged region. To quantify the possible regeneration of the spinal cord, the illuminated pixel density was calculated in the damaged region and normalized with nearby healthy spinal cord of the same mice (Figure 2B). Initially, both sham- and stem cell-treated groups presented similar values. However, the stem cell-treated mice presented progressively higher values after each passing month, these values being significant compared with sham controls at 5 months after the surgical intervention. At 6 months, the average illuminated pixel density values of the lesion compared to the adjacent spinal cord in the stem cell-treated mice were very similar. The values obtained in the sham controls, on the other hand, did not significantly change throughout the whole time of experimentation.

Immunohistochemical analysis was performed at 2, 4 and 6 months postintervention in both sham- and stem cell-treated mice. The spinal cords showed clear signs of mechanical damage in both groups of mice, including posterior horn destruction, the presence of cavities and neuronal cell loss. However histological differences were observed after 6 months between the sham- and stem-cell treated groups (Figure 2C). Whereas the spinal cord remained greatly injured in the sham controls, with little improvement, the stem cell-treated group presented a more normal anatomy.

Furthermore, the number of motor neurons observed per section was calculated in the sham- and stem cell-treated groups at the different time points (Figure 2D). As observed in the MRI analysis, after 4 months of the spinal cord lesion, a significant increase in the number of motor neurons was observed in the stem cell-treated group compared with the sham-treated group, with similar numbers observed after 6 months of the lesion. The sham-treated group presented an increased number of motor neurons at a slower rate, being equal to the stem-cell treated group after 6 months of the lesion. These results seemed to indicate that the stem cell treatment accelerated motor neuron repair.

Neurotrophic factors BDNF, NT3 & NT4 are secreted & released into the muscle tissue by the grafted stem cells

At 2, 4 and 6 months after the surgical intervention, the hindlimb muscles where stem cells were injected were analyzed by PCR to confirm the presence of the grafted cells (Figure 3A). This data was corroborated by immunohistochemistry, where the coexpression of several trophic factors and *GFP* were also evidenced (Figure 3B). Quantitative, real time PCR indicated that the expression of neurotrophic factors *BDNF, NT3* and *NT4* were upregulated in the muscle tissue where stem cells were grafted (Figure 3C). Other factors were also analyzed, but only the three previously mentioned were upregulated compared with sham controls (data not shown). The three factors are known to be implicated in neuronal survival, repair and regeneration, thus they are possible candidate factors that may be retrogradely transported into the motor neurons and inducing the regeneration processes observed in the spinal cord. Our previous works have demonstrated that bone marrow-derived mesenchymal stem cells are capable of producing and secreting *BDNF, NT3* and *NT4*, and can increase their local expression in response to adverse conditions affecting the nervous system [43].

Muscle regeneration & protection is enhanced by stem cell injection

SCI includes motor neuron cell death by direct mechanical and neurotoxic effects derived from post-traumatic hypoxia and inflammation, which eventually causes muscle atrophy in the deinnervated fibers and overall muscle tissue degeneration. Thus, muscle regeneration and survival markers were analyzed in the hindlimb muscle tissues that were treated with mesenchymal stem cells. A total of eight genes were analyzed, comparing wild-type and sham controls of the same ages (Figure 3D). The sham-operated mice presented a significant downregulation of almost all the muscle-regenerating related genes, with the exception of *MEF2C* and myogenin *(MYOG)*. This indicated a significant degenerating process in the muscle tissue and limited regeneration, which lasted throughout the

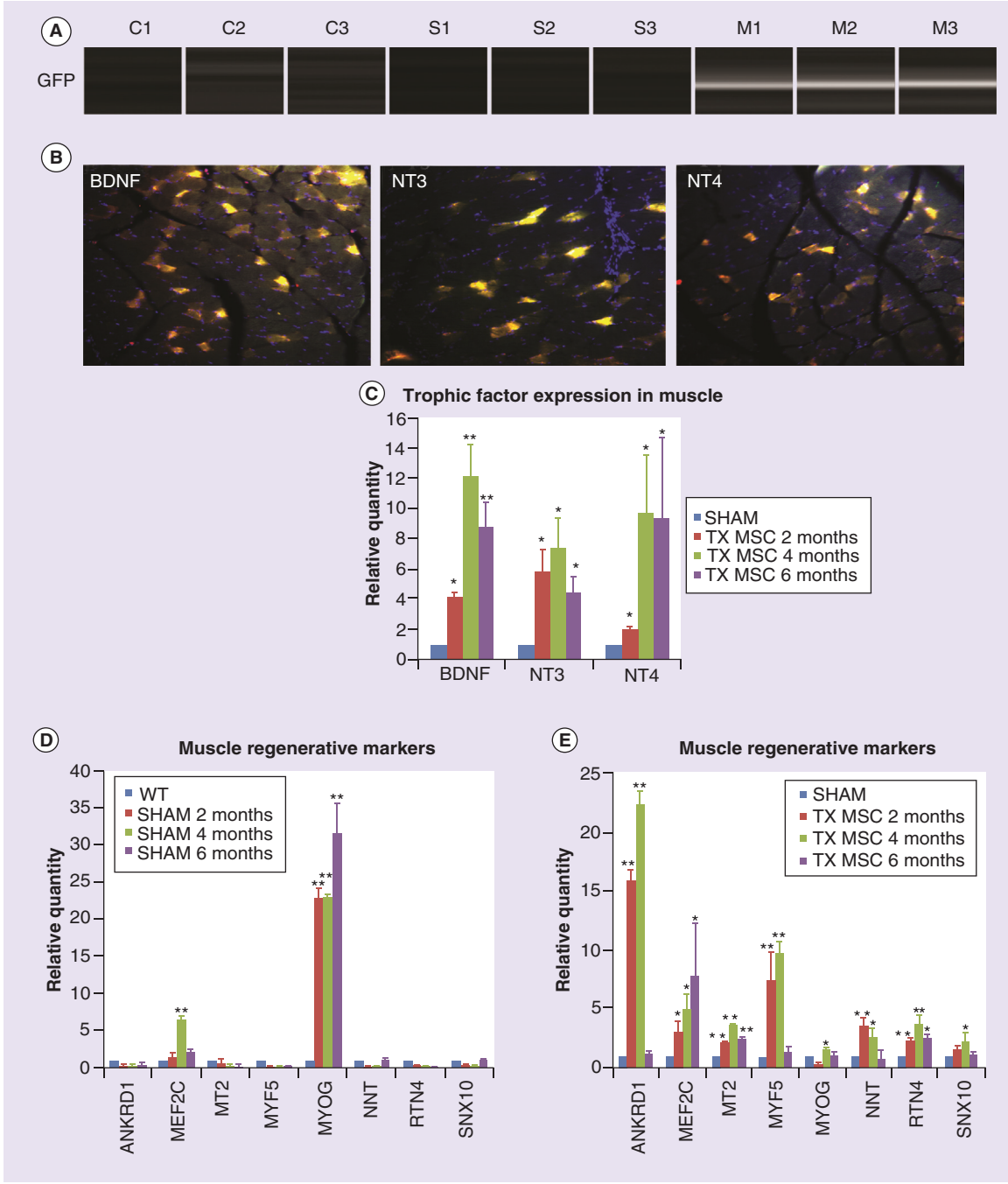

Figure 3. Analysis of the muscle tissue. (A) Conventional PCR of 3 control, 3 sham and 3 mesenchymal stem cell-treated mice analyzed for *GFP* expression, performed 6 months after the surgical intervention in the case of the sham and stem cell-treated groups. **(B)** Immunohistochemical analysis of the hindlimb muscle tissue in mice treated with GFP-expressing mesenchymal stem cells. *GFP* is shown in green, the trophic factors *BDNF, NT3* and *NT4* in red, and nuclei stained with DAPI. Images taken at 100×. **(C)** Quantitative PCR analysis of the expression of trophic factors *BDNF, NT3* and *NT4* in sham and stem cell-treated mice at 2, 4, and 6 months after the surgical intervention. The stem cell-treated groups were compared with the sham-operated group of their respective time point (sham 2 months vs TX MSC 2 months, etc). **(D)** Quantitative PCR analysis of the expression of muscle regeneration markers in wild-type and sham-operated mice at 2, 4 and 6 months after the surgical intervention. **(E)** Quantitative PCR analysis of the expression of muscle regeneration markers in sham- and stem cell-treated mice at 2, 4 and 6 months after the surgical intervention. The stem cell-treated groups were compared to the sham-operated group of their respective time point (sham 2 months vs TX MSC 2 months, etc). n = 4 in each group.
*p < 0.05; **p < 0.01.
For color images please see online www.futuremedicine.com/doi/full/10.2217/rme.14.38

whole time of experimentation. *ANKRD1* is implicated in muscle plasticity; *MEF2C, MYF5* and *MYOG* are implicated in muscle regeneration processes; *MT2* is related to oxidative stress in the muscle and muscle atrophy; nicotinamide nucleotide transhydrogenase (NNT) and *SNX10* are related to muscle metabolism; and finally, *RTN4* is related to deinnervation [48].

However, the expression pattern of these genes greatly differed in the mesenchymal stem cell-treated mice compared with the sham controls (Figure 3E). All the analyzed genes were upregulated compared with sham controls after 2 months of the intervention in the stem cell-treated groups, which continued at 4 months. The increased expression of these genes indicated an induction of regenerative processes in the muscle fibers, which was observed in the muscles where stem cells were injected. At the last time point, 6 months, the expression of almost all the genes analyzed lowered to standard levels (in many cases similar to wild-type values, data not shown), possibly indicating a finalization of the active regenerative process and normalization of the muscular tissue.

Discussion

In this work we have demonstrated that mesenchymal stem cells can accelerate neural regeneration after an acute, incomplete spinal cord lesion when grafted into the skeletal muscle. This in turn allowed the treated mice to significantly improve in their motor skills, obtaining better scores as early as 5 months after injection, while control mice did not present signs of improvement at any moment during the experimental procedure. Previous studies in our laboratory demonstrated this property in a neurodegenerative motor neuron mouse model [40], where the bone marrow stem cells, when transplanted into hindlimb muscles, secreted trophic factors that were captured by the motor end plates and retrogradely transported into the spinal cord. The expression of known soluble factors that are involved in neural regeneration and protection, such as *BDNF, NT3* and *NT4*, were detected by the grafted stem cells. Mesenchymal stem cells are known to upregulate the expression and increase secretion of these trophic factors under adverse conditions, as a paracrine protective effect [43].

One of the aspects that was observed in this work was an increase in the number of motorneurons in the spinal cord throughout the time of experimentation (Figure 2C & D). This was detected both in the sham- and stem cell-treated mice; however, there was a higher number of motor neurons in the stem cell-treated mice compared with the sham controls at 4 months. At 6 months, both groups presented similar values. This observation seems to indicate that the type of injury performed, which is an incomplete lesion by compression, allows for a certain degree of spontaneous repair in the spinal cord. Indeed, the fact that fewer motor neurons were detected by immunohistochemistry at the earliest time point, 2 months, followed by an increase in number at 4 and 6 months is most probably due to repair mechanisms of damaged motor neurons, and not neurogenesis.

Besides an improvement in motor skills and partial neural recovery, an increase in the expression of molecules involved in muscle regeneration and protection was detected. Spinal cord lesion causes muscle denervation and ultimately muscular atrophy, thus skeletal muscle-protective markers, as well as those implicated in regeneration, are important aspects to consider. The sham-operated mice presented a downregulation of almost all the genes analyzed related to muscle regeneration, with the exception of *MYOG* and *MEF2C*. This may indicate that the hindlimb muscles were undergoing degenerative processes, quite possibly due to deinnervation. In turn, mesenchymal stem cell-injected mice presented increased levels of expression of all the myo-regenerative genes analyzed, indicating a local effect, besides a direct or indirect effect on the spinal cord.

Several trophic factors were analyzed in the muscle tissue, with *BDNF, NT3* and *NT4* being the most highly expressed. Other factors included epidermal growth factor (EGF), fibroblast growth factor 2 (FGF2), insulin-like growth factor (IGF), GDNF and nerve growth factor (NGF), but none presented increased expression values compared with the sham-operated mice (data not shown). These three trophic factors are known to induce neuroregeneration in the damaged spinal cord both *in vitro* and *in vivo*, and have been linked to motor function improvement [49,50]. Furthermore, there is a previous study demonstrating that retrograde delivery of BDNF from the skeletal muscle by adenovirus-mediated gene delivery exerted a neuroprotective and oligodendrocytic-protective effect in a genetic mouse model of spontaneous spinal cord compression [51]. Our laboratory has also proven that bone marrow stem cells are capable of this effect in another genetic motor neuron degenerative model [40].

There are many published reports demonstrating the beneficial effects of bone marrow-derived stem cells in SCI, even resulting in significant functional recovery [8–23]. In several of these works, the majority of which have been performed in rats, functional recovery was detected as early as 1–2 months after treatment, compared with the 5 months detected in this study. Few of these studies, however, have analyzed the effect of the treatment at the time frame used here, where the mice were studied up to 6 months after treatment. Thus, the long-term effect

of the treatments analyzed in these studies cannot be known. Furthermore, these works have mainly focused on direct stem cell injection into the spinal cord, which is a potentially dangerous surgical intervention that may cause further damage to the tissue. Other methods of administration that have been used include intravenous administration or lumbar puncture [52–54]. These two methods have the advantage of eliminating the risk of further tissue damage, and the cells are capable of migrating to the lesion where they exert their effect. However, these approaches have the disadvantage of low percentage of engraftment, with less than 4% of the cells migrating and integrating into the lesion site [55]. Thus, for these methods to be effective it is necessary to inject a large number of stem cells, which may be a handicap as the stem cells must be harvested and expanded in culture until the appropriate cell number is reached. This is very relevant when considering these approaches be used during the acute stage of the lesion, when repair and regeneration mechanisms are possible.

As previously commented, there are several studies, including from our lab, that have shown that bone marrow-derived stem cells are capable of exerting similar effects when grafted into the hindlimb muscles [39,40], by releasing soluble factors that are captured by the motor-end plates and transported to the motor neuron cell body in the spinal cord. These works, which are a similar approach as with intravenous or intrathecal injections [52–54] without the low level of engraftment, have indicated that this approach is potentially even more effective than direct injection to the spinal cord, as it avoids the potential puncture damage, as well as protects not only the motor neuron but its axonal projection as well. In this work, of the trophic factors secreted, *BDNF* is of utmost importance as it has been shown to induce nerve growth and overall neuronal survival [18]. In fact, exogenous administration of *BDNF* has been shown to promote regeneration of sensory neurons after SCI [56]. Thus, the mesenchymal stem cells may be acting as a biological pump that releases this trophic factor to the surrounding tissue, which is captured by the motor-end plates and retrogradely transported to motor neurons, and ultimately activating repair/regeneration mechanisms.

In conclusion, bone marrow-derived mesenchymal stem cell injection into the hindlimb muscles in a mouse model of thoracic incomplete, acute SCI is capable of enhancing spinal cord repair, ultimately resulting in motor function improvement 5 months after the surgical treatment. Owing to the relative simplicity and little invasive method of the approach, this is a feasible method to use in the clinic to treat incomplete spinal cord lesions in humans soon after injury, either by using autologous (bone marrow extraction and several weeks of culture to increase stem cell number, or directly by cell-sorting the adequate cell population) or allogeneic-derived stem cells.

Future perspective

Numerous studies demonstrate that bone marrow-derived stem cells present a potentially beneficial effect on SCI. However, in the clinic this has been met with limited success, which is possibly, at least partially, due to the method of administration, as the stem cells have been directly injected into the lesion site. This may cause further damage, hampering their efficacy. Previous results using motor neuron degenerative mouse models have shown that bone marrow

Executive summary

Stem cell injection into the hindlimb muscles improves motor functions in mice with spinal cord injury

- The mice treated with bone marrow-derived mesenchymal stem cells presented significantly better scores in the treadmill (maximum running speed) and footprint (longer stride lengths) tests.
- The non-treated mice, on the other hand, did not improve in any of the tests performed throughout the whole time of experimentation.

Mesenchymal stem cells, when grafted into the muscle tissue of mice with spinal cord injury, release numerous soluble factors that induce repair mechanisms

- The spinal cords of the stem cell-treated mice presented signs of accelerated repair mechanisms, analyzed both by MRI and immunohistochemistry.
- This was due to the release of trophic factors, such as BDNF, NT3 and NT4, which are known to activate prosurvival mechanisms and regeneration.

Muscle regeneration & protection is enhanced by stem cell injection

- After spinal cord injury, there is a deinnervation of the muscles, which ultimately causes muscle atrophy.
- In our experiments, upregulation of muscle regeneration and repair markers was detected in the hindlimbs of the stem cell-treated mice, compared with non-treated sham controls.
- This indicates the presence of a local repair effect of the stem cells in the hindlimb muscles besides the retrograde effect in the motor end plates.

stem cells can exert similar effects when transplanted into the skeletal muscle tissue, by releasing trophic factors that are captured by the motor-end plates that innervate the muscle. The factors then travel retrogradely to the motor neuron cell body, activating prosurvival mechanisms while improving motor functions. The work presented in this study demonstrates that it is possible to extrapolate this surgical method into an acute SCI model. Due to the feasibility of the approach, the little invasive and low risk that it presents, this work may allow, in the next few years, clinical trials administering intramuscularly bone marrow-derived stem cells in acute spinal cord lesions to induce repair and/or regeneration.

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Ethical conduct of research

The authors state that they have obtained appropriate institutional review board approval or have followed the principles outlined in the Declaration of Helsinki for all human or animal experimental investigations. In addition, for investigations involving human subjects, informed consent has been obtained from the participants involved.

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