

Mesenchymal stem cell treatment of intervertebral disc lesion prevents fatty infiltration and fibrosis of the multifidus muscle, but not cytokine and muscle fiber changes

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Abstract

Study Design. Longitudinal case control animal model.

Objective. To investigate effects of mesenchymal stem cell (MSC) treatment on multifidus muscle remodeling after intervertebral disc (IVD) lesion.

Summary of Background Data. Lesion and degeneration of IVDs causes structural remodeling of the multifidus muscle. Pro-inflammatory cytokines are thought to contribute. MSC-treatment restores IVD health after lesion but its effects on surrounding tissues remains unknown. Using an animal model of IVD degeneration, we assessed the effects of MSC-treatment of IVDs on the structural remodeling and cytokine expression within the multifidus muscle.

Methods. An anterolateral lesion was performed on the L1-2, L3-4 and L5-6 IVDs in sheep. At either 4 (early treatment) or 12 (late treatment) weeks after IVD lesion, MSCs were injected into the lesioned IVD. Multifidus muscle was harvested from L2 (gene expression analysis) and L4 (histological analysis) at 3 or 6 months after IVD lesion and naïve controls for histological analysis of muscle, adipose and connective tissue cross sectional areas (CSA), and immunohistochemistry to study muscle fiber types. Real-time polymerase chain reactions quantified expression of TNF, IL-1 β and TGF- β 1.

Results. MSC-treatment of IVD lesion prevented the increased adipose and connective tissue CSA expected after IVD lesion. MSC-treatment did not prevent slow-to-fast muscle fiber type transformation. Gene expression of pro-inflammatory cytokines within the muscle was altered by the MSC-treatment of IVD. Increased IL-1 β expression was prevented in the early treatment group and TNF and TGF- β 1 expression was upregulated at 6 months.

Conclusion. Results show that although MSC-treatment prevents fatty infiltration and fibrosis of the multifidus muscle after IVD lesion, it cannot prevent a muscle inflammatory response and muscle fiber transformation. These findings highlight the potential role of MSC

therapy after IVD injury, but reveals that other interventions may also be necessary to optimize recovery of muscle.

Key Words. Mesenchymal stem cell, multifidus, adipose, connective tissue, hypertrophy, atrophy, intervertebral disc degeneration, cytokine, fatty infiltration, fiber type transformation

Level of Evidence: 4

ACCEPTED

Introduction

Low back pain (LBP) is the leading cause of disability internationally^{1,2} and is commonly associated with intervertebral disc (IVD) disease^{3,4} and structural⁵⁻¹⁰ and behavioral¹¹⁻¹⁴ alterations to the multifidus muscle. Experimental IVD injury causes rapid atrophy¹⁵ and inhibition¹⁶ of multifidus within days, then later structural remodeling (e.g. fatty infiltration, fibrosis)¹⁷. Such changes have relevance for spine control and pain^{18,19}. Stem cell therapies for IVD injury/degeneration have promising results for prevention/restoration of IVD properties²⁰. Whether these treatments also influence the health of surrounding tissues, including back muscles, remains unknown.

In acute LBP, reduced spinal excitability¹⁶ mediates reduced cross-sectional area (CSA) of multifidus⁵. MRI analysis in chronic LBP reveals multifidus fatty infiltration⁷⁻⁹ and atrophy²¹, potentially related to disuse. Animal studies reveal a temporal pattern of multifidus remodeling following induction of IVD disease characterized by increased adipose²² and connective tissue^{22,23}, slow-to-fast muscle fiber type transformation^{17,22}, but no changes in whole muscle/muscle fiber CSA²². Muscle remodeling in the intermediate period appear mediated by an inflammatory response involving upregulated pro-inflammatory cytokines gene expression (e.g. tumor necrosis factor [TNF], interleukin 1beta [IL-1 β]²²). This suggests LBP-induced muscle changes are mediated by different mechanisms and time courses; each with relevance for spinal health.

Mesenchymal stem cell (MSC) treatment is a promising IVD therapy^{20,24}; it both prevents and repairs IVD degeneration, depending on the time of treatment²⁴. Whether MSC IVD treatment also impacts health of surrounding tissues, including multifidus, and if this differs with time of application but has not been determined.

This study aimed to investigate the effect of MSC application into an injured IVD on structural and inflammatory changes within multifidus. We studied whether MSC-treatment

prevents (early MSC application) or restores (late MSC application) multifidus remodeling after experimental IVD lesion. Further, we investigated whether early/late MSC-treatment modified pro-inflammatory cytokine gene expression within the muscle.

Materials and Methods

Animals

Forty-five merino wethers (aged 3-4 yrs) as part of a study investigating MSC-treatment on IVD lesions were used²⁴. All procedures were carried out with the approval of the institutional animal care and ethics committee.

Surgical procedure, IVD lesion, MSC injection, postoperative care and tissue harvesting

Ten age- and weight-matched sheep which did not undergo a surgical procedure, receive an IVD lesion or an MSC injection were assigned to the Control group (histological analysis of the multifidus muscle of these animals has been reported previously²²). Thirty-five animals underwent surgery as previously described¹⁷. Briefly, an extraperitoneal surgical approach was used to induce a 20 mm wide x 6 mm deep annulus fibrosis lesion (without penetrating the nucleus pulposus) on the left anterolateral region of IVDs at L1-2, L3-4 and L5-6 (Fig. 1A). This lesion induces progressive disc degeneration mimicking many of the pathological and molecular changes in patients²⁵. For ten days post-surgery, animals were closely monitored in pens to ensure wound healing, absence of gait abnormalities or surgical complications. At four (Early treatment group; n=24) or twelve (Late treatment group; n=12) weeks after the initial surgery, half of each group of animals received MSC (10^7 culture-expanded heterologous bone marrow derived ovine cells/0.2 ml PBS²⁴ which were selected as a clinically relevant cell source that has been evaluated for a variety of therapeutic applications particularly for applications related to stimulation of chondrogenesis²⁶) or PBS carrier (Untreated injured group – histological analysis of the multifidus muscle of these animals has been reported previously²²) injected into the nucleus pulposus of the operated

IVD via the right side under direct surgical visualization (Fig. 1B). All animals were co-housed in an open paddock with unrestricted exercise. Multifidus muscle (Fig. 1C and Fig. 1D) was harvested at 3 (n=6: Early treatment/Untreated; n=4 Control) or 6 (n=6: Early or Late treated/Untreated; n=6 control) months after the IVD lesion and processed as previously described¹⁷ (Fig. 1G).

Analysis of muscle, connective and adipose tissues

Paraffin-embedded multifidus muscle from L4 (lesion, contralateral) were sectioned (8μm), their muscle, connective and adipose tissue composition analyzed using Masson's trichrome and Van Gieson's stains (Fig. 1E). Entire multifidus sections were imaged (Scanscope AT Turbo, Aperio, Vista, CA). Each tissue type (adipose, muscle, connective tissue) was identified based on color²² (Fig. 1F) and CSAs were quantified using ImageJ (NIH, Bethesda, MD)²².

Muscle fiber-type analysis

The immunohistochemistry assay was performed as previously described¹⁷ using reagents in Table 1. Whole sections of multifidus were imaged (ScanScope AT Turbo, Aperio, Vista, CA) and muscle fiber type proportion and CSA were quantified. Multifidus was divided into 36 regions¹⁷, with two images taken within each region. From each image, the proportions of slow (stained black/brown), fast (stained red/pink) and intermediate fibers (combination of slow and fast) were determined based on staining colour and intensity (Fig. 1F)¹⁷. CSA of 5-6 randomly chosen fibres of each type was quantified using ImageJ (NIH, Bethesda, MD)²².

Cytokine gene expression

Total RNA was extracted from the deepest fascicle of multifidus at L2 using an RNeasy Mini Kit (Qiagen, Hilden, Germany). RNA was reverse-transcribed with the Super Script III first strand synthesis kit (Invitrogen, Carlsbad, CA) and real-time polymerase chain

reaction preformed using the IQ SYBR Green Mastermix (Bio-Rad Laboratories, Hercules, CA). Gene expressions were first converted to a percentage of the housekeeping gene GAPDH then to fold difference to the mean of the control values. Primer pairs used in this experiment are listed in Table 2).

Statistical analysis

All measures were compared between sides (injured/left vs. uninjured/right; within subject factor) and groups at 3 (Control vs. Untreated injured vs. Early treatment) and 6 (Control vs. Untreated injured vs. Early and Late treatment groups; between subject factor) months post-injury with separate repeated measures analysis of variance (ANOVA). Post-hoc analysis involved Duncan's multiple range test. Comparison of Control to Untreated injured animals has been reported earlier²². Data are expressed as mean±SD. Significance was set at $P<0.05$.

Results

Whole muscle, connective and adipose tissue analysis

Early treatment group – 3 months

Table 3 presents statistical analysis of Early MSC-treatment at 3 months. In contrast to the previously reported²² lack of difference between Control and Untreated injured for any measure, connective tissue CSA was less after Early MSC-treatment than Control and had a non-significant tendency to be less than Untreated injury (post hoc $P=0.058$) on both sides (Fig. 2A). Muscle and adipose tissue CSA did not differ between MSC treated and Control/Untreated injured (Fig. 2A).

Early treatment group – 6 months

Table 4 presents statistical analysis of 6-month data for Early and Late MSC-treatment. MSC-treatment during the acute phase of IVD degeneration prevented the increased in CSA of connective tissue observed in Untreated injured relative to Control²² (Fig. 3A). Connective tissue CSA was less for the MSC-treatment than Untreated injury group. MSC-treatment induced multifidus hypertrophy on both sides relative to Control/Untreated injury (Fig. 3A).

Late treatment group – 6 months

MSC-treated sheep at 12 weeks would already have established IVD degeneration²⁴. Yet, muscle, adipose or connective tissue CSA of muscle harvested at 6 months was not different between Late MSC treated and Control (Fig. 3A). This contrasts with increased adipose and connective tissue CSA in untreated animals²² (Fig. 3A). Late connective tissue CSA was less than Untreated injured. Muscle CSA was smaller for the Late than Early treated group at 6 months.

Muscle fiber analysis

Early treatment group – 3 months

As for untreated injured²² Early MSC-treatment did not change the proportion of slow, fast and intermediate muscle fibers relative to Control and Untreated injured (Fig. 2B). CSA of slow or fast muscle fibers did not differ between groups, but intermediate muscle fiber CSA was less for Early treatment (Fig. 2D).

Early treatment group – 6 months

At 6 months Early MSC-treatment did not prevent muscle fiber changes induced by IVD lesion²². The proportion of slow fibers was reduced on both sides at 6 months relative to

Control (Fig. 3B), and not different to Untreated injured. Intermediate fibers increased after Early MSC-treatment relative to Control and Untreated injured. The proportion of fast fibers was unchanged by Early MSC-treatment although this had been reported in Untreated injury²². There was no difference in CSA of slow or intermediate muscle fibers (Fig. 3D), but fast fibers were smaller on the injured side for all groups.

Late treatment group – 6 months

Late MSC-treatment had a lower proportion of slow fibers than Control and no difference to Untreated injured on both sides (Fig. 3B). The proportion of intermediate fibers was greater for the Late treatment group than Control and Untreated injured for the injured side, but only the Control animals on the non-injured (right) side. Proportion of fast fibers was not different between Late MSC treated and other groups. CSA of muscle fibres were unchanged for slow and intermediate muscle fibers, but smaller for the fast fibers on the injured side for all groups (Fig. 3D).

Pro-inflammatory cytokine gene expression

Early treatment group – 3 months

Early MSC-treatment did not alter the expression of TNF or TGF- β 1 at 3-months relative to Control/Untreated injured. IL-1 β expression was less on both sides of the MSC treated animals than Control/Untreated injured (Fig. 2C).

Early treatment group – 6 months

At 6 months, the Early MSC-treatment group had greater TNF expression than Control animals on the injured but not non-injured side (Fig. 3C), and was greater than the increased previously observed for Untreated injured²². TGF- β 1 expression was greater on the injured side of the Early MSC treated than Untreated injured animals. IL-1 β expression in the Early MSC-treated group was similar to Control/Untreated injured (Fig. 3C). Increased TNF

and IL-1 α expression in Untreated injured relative to controls²² narrowly missed significance unlike the previous study that retained higher power due to fewer analyses.

Late treatment group – 6 months

Late MSC-treatment increased TNF expression on both sides relative to Controls and on the injured side relative to Untreated injured (Fig. 3C). Late MSC-treatment had a tendency (P=0.059) for greater TGF- β 1 expression than Control on the injured side only.

Expression of IL-1 β was not different between groups.

Discussion

Treatment of injured IVD (early or late) has positive potential to prevent or restore structural remodeling of adipose and connective tissue of adjacent muscles, but the timing of treatment is important. MSC-treatment modified the muscle's inflammatory response but did not prevent, and in the Late treatment group actually increased TNF expression or the transformation of muscle fibers induced by IVD injury. This will impact the muscle's functional capacity and suggests MSC-treatment alone cannot prevent/reverse all effects of IVD lesion.

Mechanisms for MSC-treatment of IVD to affect muscle changes

MSC-treatment of IVD lesion not only affects the injured IVD²⁴, but also surrounding muscles. Although MSC differentiation into various cell types has been considered the primary role in treatment of musculoskeletal disorders, recent research suggests a role of paracrine effects²⁷. Paracrine signaling factors secreted from MSCs have anti-fibrotic, immunomodulatory and anti-inflammatory effects in surrounding tissues^{28,29}. MSCs interact with immune cells such as polarizing macrophages from a pro-(M1) to anti-inflammatory (M2) state^{30,31}. M1 macrophages express high levels of pro-inflammatory cytokines (TNF/IL-1 β). M2 macrophages express TGF- β 1 in an anti-inflammatory role³², but undetectable IL-1 β ^{33,34}. MSC-induced polarization towards anti-inflammatory M2 macrophages in multifidus observed here could explain reduced IL-1 β expression in Early treated animals and increased TGF- β 1 at 6 months. Upregulated TNF expression indicates MSC-treatment did not dampen the pro-inflammatory response of TNF after IVD lesion. Alternatively, positive effects of MSC-treatment of IVD on muscle physiology might be an indirect consequence of improved muscle activation secondary to improved IVD health. Muscle loading/unloading has potent effects on fatty infiltration³⁵, fibrosis³⁶ and muscle fibers³⁷ and requires consideration.

MSC-treatment prevents increased adipose tissue

Fatty infiltration is common in back muscles, particularly multifidus, in LBP^{7,9}, and develops by 6, but not 3, months, on the injured side after IVD injury in sheep²². Although mechanisms are poorly understood, data from humans and animals imply rapidly developing adipogenesis^{15,23} recovers^{38,8}, with subsequent gradual development of sustained fatty infiltration over 3-6 months²². Our data indicate MSC-treatment of an injured IVD either Early (preventing IVD degeneration) or Late (restoring IVD degeneration) prevents fatty infiltration, suggesting ongoing IVD health is more important than initial injury for driving fatty infiltration.

TNF³⁹ and TGF- β 1⁴⁰ regulates adipocyte development from either progenitor MSC⁴¹ or trans-differentiation from myoblasts⁴². TGF- β 1 antagonizes adipogenesis⁴⁰, whereas TNF has both adipogenic or anti-adipogenic effects depending on concentration, time-point and receptor expression⁴³. Although upregulated TNF expression paralleled increased adipose CSA in untreated animals²², this study showed upregulation of both TNF and TGF- β 1 following MSC-treatment. Thus, either pro-adipogenic effects of TNF were antagonized by increased TGF- β 1 expression or MSC-treatment changed TNF to an anti-adipogenic function. Muscle unloading reduces expression of factors that inhibit myoblast transdifferentiation to adipocytes³⁵, thus maintained muscle activation by improved IVD health might explain maintained adipose CSA.

MSC-treatment prevents increased connective tissue

MSC-treatment of the IVD prevented the accumulation of connective tissue in multifidus after untreated IVD injury²². Muscle fibrosis accompanies muscle injury for muscle repair⁴⁴ and increases with ageing⁴⁵. Profibrotic cells trans-differentiate from myogenic cells⁴⁶ when TGF- β 1 increases⁴⁴. This appears inconsistent with our data of unchanged connective tissue despite increased TGF- β 1, but could be explained by complex interplay between multiple cytokines and differing temporal expression (e.g. connective tissue reduced at 3, but not 6, months after Early treatment). Anti-fibrotic effects of MSC-treatment of IVD were accompanied by an acute short-term downregulation of IL-1 β (suggesting its anti-inflammatory effect). The subsequent loss of IL-1 β -mediated inhibition of TGF- β 1⁴⁷ between 3 and 6 months may explain upregulation of TGF- β 1 by 6 months, and the recovery of connective tissue. Restoration of connective tissue CSA to control levels at 6 months may represent accelerated fibrosis in response to increased TGF- β 1 expression, but from a reduced baseline at 3 months. Improved muscle activation could also explain the absence of fibrosis potentially through muscle unloading³⁶.

MSC-treatment does not prevent muscle fiber transformation, but causes early hypertrophy

Despite promising effects on muscle fat and fibrosis, MSC-treatment of injured IVD did not prevent muscle fiber type transformation. However Early treatment augmented multifidus. Contrary to clinical predictions^{5,6,10}, earlier data of untreated IVD lesions²² show no loss of muscle CSA (whole muscle or muscle fiber) by 6 months. This concurs with human imaging within 3 months⁸ and muscle fiber histology in rabbits²³ after IVD injury. This does not preclude very early (within days^{5,15}) and late (>6 months^{6,10}) muscle atrophy²². Multifidus hypertrophy after Early, but not Late treatment has several explanations. First, MSCs regulate muscle atrophy/hypertrophy⁴⁸. MSC-treated culture media prevent muscle

atrophy through growth factor secretion⁴⁸, which could promote hypertrophy. Second, IL-1 β inhibits the growth factor pathways⁴⁹. Down-regulated IL-1 β expression at 3 months after Early treatment would reduced IL-1 β mediated inhibition of the growth factor pathway leading to its over-activation and hypertrophy. Untreated IVD injury upregulates genes in the growth factor pathway²² but did not induce hypertrophy possibly via antagonist effects of TNF on signaling molecules (e.g. Protein Kinase B(Akt1); Phosphoinositide 3-kinase(PI3k)⁵⁰). TNF effects may be modified after MSC-treatment by interaction with other cytokines.

As individual muscle fiber CSA did not increase, hypertrophy must involve increased fiber number potentially mediated by differentiation of multipotent stem cells into muscle fibers. Improved IVD function mediated by MSC-treatment is unlikely to explain hypertrophy as this would be expected to maintain, but not hypertrophy the muscle.

Mechanisms including pro-inflammatory cytokine expression²² and muscle loading⁵¹ regulates transformation, which is implied by increased intermediate fibre proportion. Slow fiber proportion potentially reduces by transformation⁵² or selective slow fiber loss⁵³ and fast fiber proliferation⁵⁴. MSC-treatment did not prevent/reverse muscle fiber transformation following IVD nor prevent upregulated TNF expression at 6 months, which coincided with fiber type transformation. This remodeling persisted despite restored IVD health at 6 months²⁴. Muscle-synthesized TNF promotes preferential fast fiber differentiation⁵⁵ and TGF- β 1 causes myotubules to preferentially develop into fast fibers⁵⁴. Parallel time courses suggest such a cytokine-driven process. Data of untreated animals also imply independence of IVD pathology and muscle changes, as IVD pathology (disc height, biomechanics, histopathology, and biochemistry) is maximal at 3 months²⁴ yet muscle changes are not apparent until 6 months¹⁷ in sheep²² and rabbits²³. Failure of MSC-treatment to prevent this inflammatory process, despite IVD healing, could explain our data.

Fast heavy chain myosin genes undergo increased transcription in absence of muscle stretch/force^{37,56}. Although MSC-treatment may restore IVD biomechanics²⁴, the pattern of multifidus stretch/load may be modified (as in persistent LBP¹¹), causing muscle fiber transformation. Reduced slow muscle fiber proportion has functional consequences for spine health. Loss of fatigue-resistant fibers could limit the capacity to sustain activity required for ongoing spine control⁵⁷.

Implications

These data highlight the potential of MSC-treatment of IVD lesion. However, not all structural remodeling was overcome by restored IVD health. Prevention of fatty infiltration and fibrosis during the sub-acute/early chronic phase implies widespread effects of MSC-treatment to surrounding tissues. Persistent upregulation of pro-inflammatory cytokine expression in multifidus and changes in muscle fiber transformation imply other interventions such as exercise or anti-inflammatory treatments may complement MSC-treatment for more complete recovery after IVD injury.

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Figure legends

Figure 1. Methods (A) Lateral view of the lumbar vertebrae (L1-6) indicating levels injured/treated (arrows). Muscle from L2 was used for histology and from L4 for PCR analysis. (B) Transverse view of an intervertebral disc (IVD), showing the locations of the annular injury and mesenchymal stem cell (MSC) injections. (C) Anatomy of the four multifidus muscle fascicles present at each level. (D) Position of four fascicles in transverse section of muscle. (E) Representative image of multifidus muscle after Van Gieson's stain. Boxed region corresponds to image in panel F. (F) Muscle sections that have undergone Van Gieson's stain (left) showing the three tissue types or muscle fiber type analysis (right) showing the slow (S, black/brown), fast (F, pink/red) and intermediate (I, combination of black & pink) fiber types. (G) Timeline of MSC administration and muscle harvesting for each of the study groups.

Fig. 1

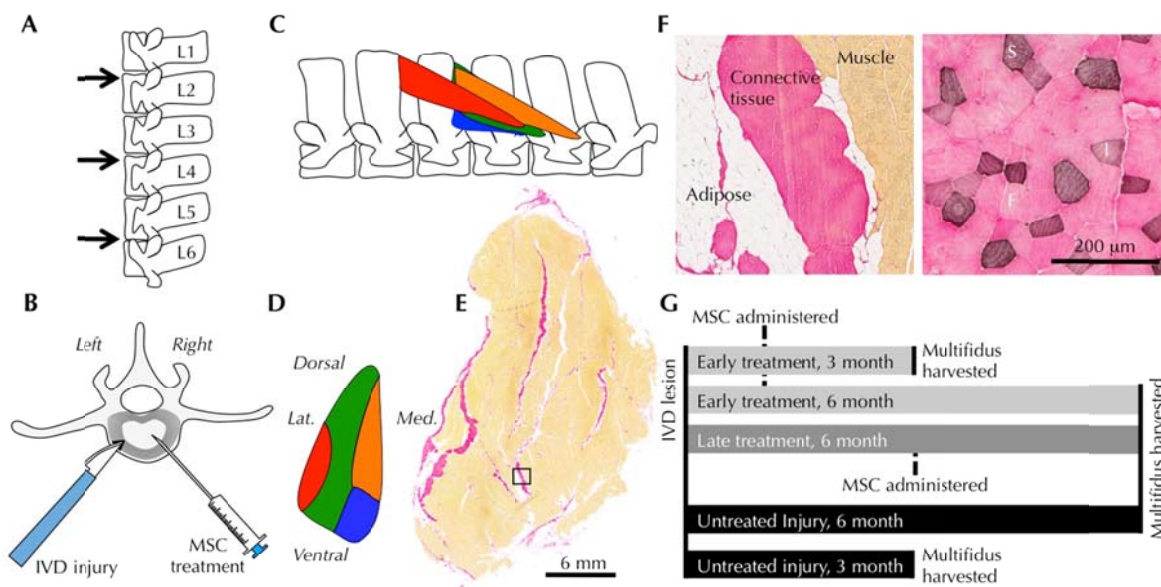


Figure 2. Effect of Early mesenchymal stem cell (MSC) treatment on structural remodelling and cytokine response at 3 months after intervertebral disc (IVD) lesion. (A) Cross sectional area (CSA) of the whole muscle, connective (Conn.) and adipose tissue. (B) Proportion (prop.) of slow, intermediate (Intermed.) and fast muscle fiber types. (C) CSA of each muscle fibre type. (D) Cytokine expression (tumor necrosis factor [TNF], interleukin 1beta [IL-1 β] and transforming growth factor-beta 1 [TGF- β 1]. Data are presented as mean +SD. * - P<0.05. I – untreated injured; NI – untreated non-injured; E3 – early MSC-treatment group at 3 months. Note the different scales for muscle vs. connective and adipose tissue CSA, and the proportion of fast vs. slow and intermed. muscle fiber types.

Fig. 2

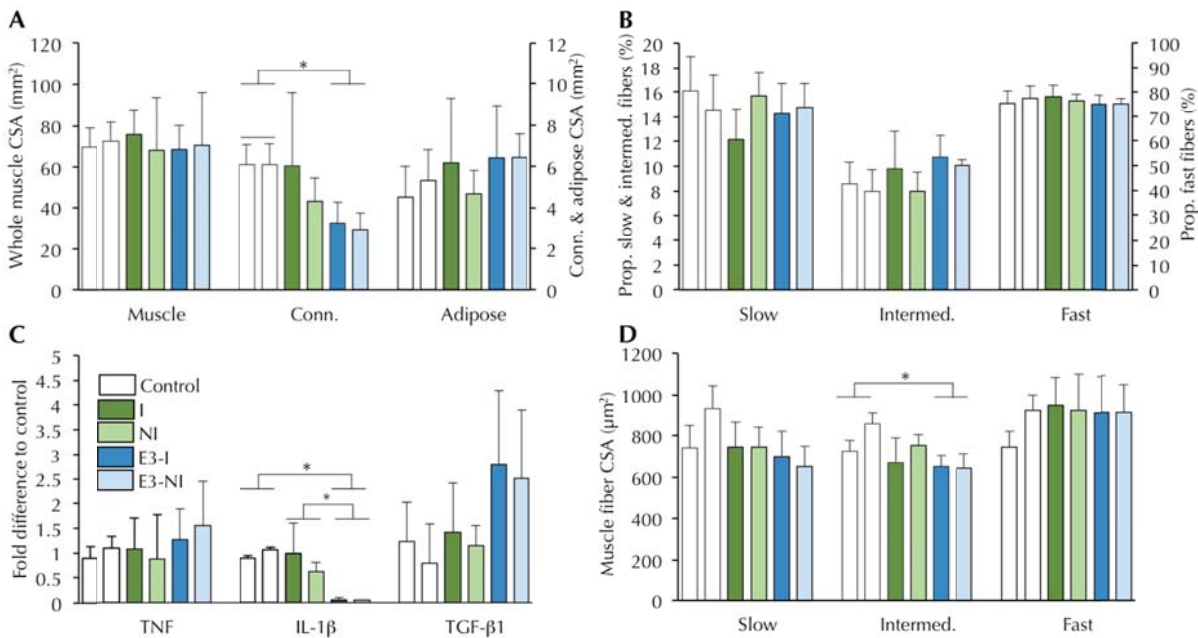


Figure 3. Effect of Early and Late mesenchymal stem cell (MSC) treatment on structural remodelling and cytokine response at 6 months after intervertebral disc (IVD) lesion. (A) Cross sectional area (CSA) of the whole muscle, connective (Conn.) and adipose tissue. (B) Proportion (prop.) of slow, intermediate (Intermed.) and fast muscle fiber types. (C) CSA of each muscle fibre type. (D) Cytokine expression (tumor necrosis factor [TNF], interleukin 1beta [IL-1 β] and transforming growth factor-beta 1 [TGF- β 1]. Data are presented as mean +SD. * - $P < 0.05$. I – untreated injured; NI – untreated non-injured; E6 – Early MSC-treatment group at 6 months; L6 – Late MSC-treatment group at 6 months. Note the different scales for muscle vs. connective and adipose tissue CSA, and the proportion of fast vs. slow and intermed. muscle fiber types. # - $P < 0.05$ for previously reported analysis of comparison between Control and Untreated injured animals²².

Fig. 3

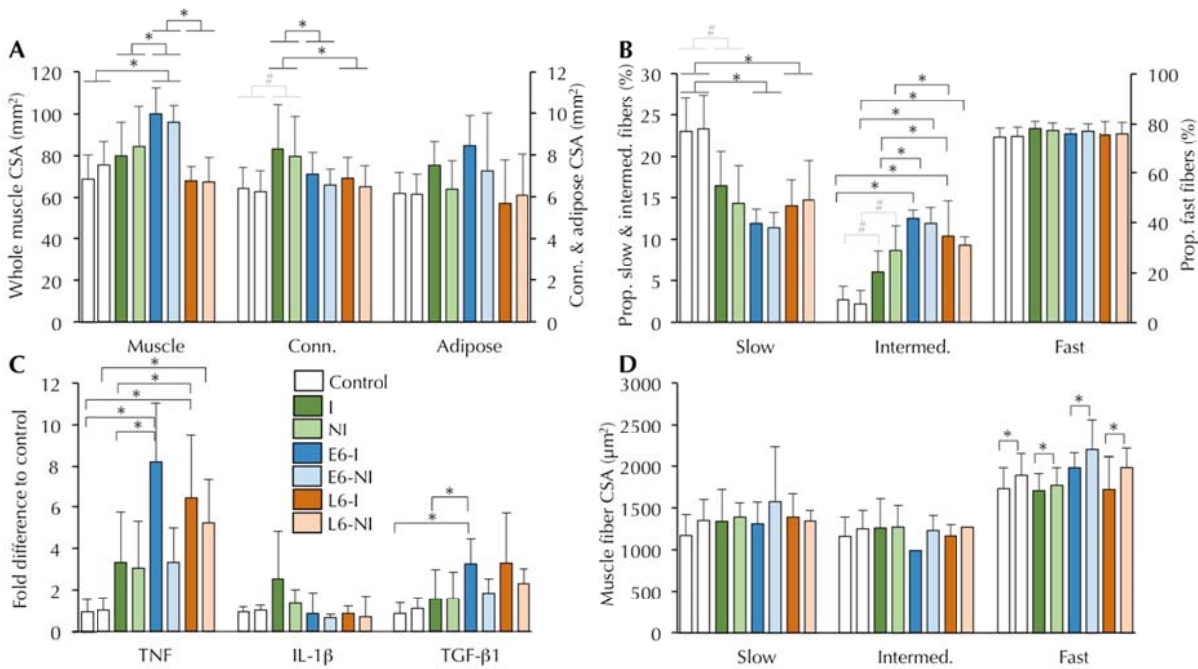


Figure 4. Representative images showing the slow-to-fast fiber type transformation after intervertebral disc (IVD) lesion, despite treatment with mesenchymal stem cell (MSC). Immunohistochemistry analysis of slow (black), fast (red/pink) and intermediate (expressing both slow and fast) in the multifidus muscle of control, Early MSC-treatment animals at 3 and 6 months and animals from the Late treatment group at 6 months.

Fig. 4

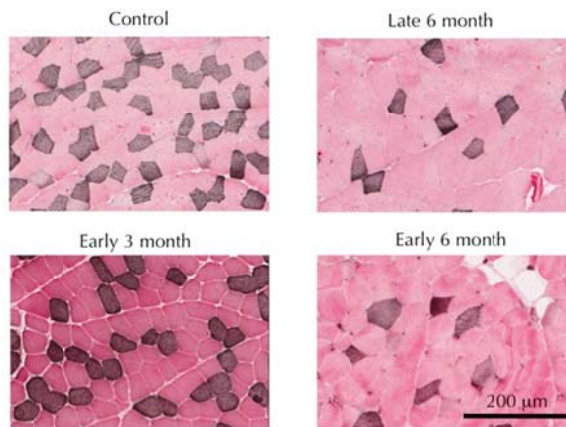


Table 1. Reagents used for muscle fiber-type analysis

Reagent	Concentration	Manufacturer
Mouse anti-Slow Myosin - NOQ7.5.4D	1:1000	Sigma, St Louis, MO
Mouse anti-fast myosin conjugated to Alkaline Phosphatase - My-32	1:50	Sigma, St Louis, MO
Rabbit anti-mouse IgG conjugated to peroxidase	1:50	Sigma, St Louis, MO
SG Peroxidase Substrate Kit	Per manufactures instructions	Vector Laboratories, Burlingame, CA
NovaRED chromagen	Per manufactures instructions	Vector Laboratories, Burlingame, CA

Table 2. Primer pairs used for real-time PCR analysis.

Gene Name	Forward Primer	Reverse Primer
TNF	5'-ACAGGCCTCTGGTTCAGACA-3'	5'-CCATGAGGGCATTGGCATAC-3'
IL-1 β	5'-CACAGGAAATGAGCCGAGAA-3'	5'-CAGCTGCAGGGTCGGTGT-3'
TGF- β 1	5'-TTACAACAGTACCCGCGACC-3'	5'-GAGCTCGGACGTGTTGAAGA-3'
GAPDH	5'-CCTGGAGAAACCTGCCAAGTATG-3'	5'-GGTAGAAGAGTGAGTGTCGCTGTTG-3'.

Table 3 Statistical analysis: Control, untreated IVD, Early mesenchymal stem cell (MSC) treatment 3-month data

	Main effect: group	Main effect: side	Interaction: group x side	Post hoc
Muscle CSA	P=0.97	P=0.86	P=0.68	
Conn. CSA	P=0.028	P=0.26	P=0.44	C > E3, P=0.013 I (>) E3, P=0.058
Adipose CSA	P=0.48	P=0.70	P=0.26	
% slow	P=0.62	P=0.76	P=0.07	
% intermed.	P=0.14	P=0.062	P=0.98	
% fast	P=0.48	P=0.25	P=0.15	
Slow CSA	P=0.32	P=0.47	P=0.056	
Intermed. CSA	P=0.046	P=0.33	P=0.57	C > E3, P=0.017
Fast CSA	P=0.82	P=0.19	P=0.17	
TNF	P=0.44	P=0.57	P=0.39	
IL-1β	P<0.001	P=0.56	P=0.22	C > E3, P<0.001 I > E3, P=0.0017
TGF-β1	P=0.067	P=0.33	P=0.99	

C – Control animals; CSA – Cross sectional area; Conn. – Connective tissue; E3 – early MSC-treatment 3 month muscle analysis; I – untreated injured animals; IL-1 β – Interleukin-1beta; TGF- β 1 – Transforming growth factor-beta1; TNF – Tumor necrosis factor; slow – slow muscle fiber; intermed. – intermediate muscle fiber; fast – fast muscle fiber

Table 4 Statistical analysis: Control, untreated IVD, early MSC-treatment 6-month data

	Main effect: group	Main effect: side	Interaction: group x side	Post hoc
Muscle CSA	P=0.0061	P=0.71	P=0.57	C<E6 P=0.0045 I<E6 P=0.031 L6<E6 P=0.013
Conn. CSA	P=0.012	P=0.20	P=0.95	I < E6 P=0.025 I < L6 P=0.019 C<I P=0.0068
Adipose CSA	P=0.12	P=0.49	P=0.66	
% slow	P<0.001	P=0.27	P=0.33	C < E6 P<0.001 C < L6 P<0.001 C>I P<0.001
% intermed.	P<0.001	P=0.65	P=0.0042	Inj side: C < E6 P<0.001 C < L6 P<0.001 I < E6 P<0.001 I < L6 P=0.019 C<I P=0.036 Non-inj side: C < E6 P<0.001 C < L6 P<0.001 I < E6 P<0.001 C<I P<0.001
% fast	P=0.35	P=0.40	P=0.51	
Slow CSA	P=0.88	P=0.22	P=0.53	
Intermed. CSA	P=0.48	P=0.12	P=0.69	
Fast CSA	P=0.10	P=0.02	P=0.65	Inj <Non-inj, P=0.02
TNF	P=0.0012	P<0.001	P<0.001	Inj side: C < E6 P<0.001 C < L6 P<0.001 I < E6 P<0.001 I < L6 P=0.019 C(<)I P=0.14 Non-inj side: C < L6 P=0.0050 C(<)I P=0.18
IL-1β	P=0.062	P=0.24	P=0.35	C(<)I P=0.094
TGF-β1	P=0.058	P=0.16	P=0.017	Inj side: C < E6 P=0.0020 C (<) L6 P=0.059 I < E6 P=0.023

C – Control animals; CSA – Cross sectional area; Conn. – Connective tissue; E6 – early MSC-treatment 6-month muscle analysis; I – untreated injured animals; IL-1 β – Interleukin-1beta; L6 – late MSC-treatment 6-month muscle analysis; TGF-b1 – Transforming growth

factor-beta1; TNF – Tumor necrosis factor; slow – slow muscle fiber; intermed. – intermediate muscle fiber; fast – fast muscle fiber

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