Gallant

# Autologous mesenchymal stromal/stem cells for equine diseases

## Introduction

For more than two decades, mesenchymal stromal/stem cells (MSCs) have been under investigation for therapeutic use in a variety of human and animal diseases.[1] Due to immune-modulating and anti-inflammatory properties, studies have evaluated MSCs for a broad variety of inflammatory and immune-mediated diseases, while investigators in the equine field have focused most of their attention on musculoskeletal conditions, predominantly superficial digital flexor tendon (SDFT) injury and chronic osteoarthritis (OA).[2-5]

This white paper provides a summary of clinical research involving autologous MSCs for equine conditions, including a brief characterization of equine adipose-derived MSCs (AD-MSCs), followed by a review of clinical trials for OA, tendon and ligament injuries, laminitis, and exertional rhabdomyolysis.

## Adipose-derived MSCs in equine medicine

In horses, AD-MSCs have shown therapeutic potential for tendon, cartilage, and bone lesions, and are therefore a common cell type under investigation and in clinical use.[6] Compared with equine bone marrow-derived MSCs (BM-MSCs), which have also been widely studied, equine AD-MSCs may be easier and safer to harvest, and exhibit more sustained self-renewal and multipotential differential (after 8 cell passages, AD-MSCs show no signs of cell senescence, whereas BM-MSCs demonstrate senescence after 7 passages).[6,7] In contrast with these attributes, AD-MSCs may exhibit inferior immunomodulatory potential, compared with BM-MSCs (and endometrium-derived MSCSs), based on relatively lower expression of MCP-1, CCL6, IL-6, and IL-8; however, this finding does come with a caveat: it may have been due to macrophage contamination of non-AD-MSC preparations used in the study.[8] Unfortunately, similar studies are currently lacking.

Equine AD-MSCs may also vary in cellular expression and behavior depending on their source. Gene expression and differentiation potency can deviate from donor to donor, depending on donor age and morbidity, and even between types of fat from the same donor. A 2019 equine study by Arnhold and colleagues, for instance, compared stemness and multipotency across MSCs derived from three types of fat: retroperitoneal (RP), subcutaneous (SC), and lipomatous (LP).[6] Results showed a variety of



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differences between the cells, including greater chondrogenic potential for RP- and SC-MSCs versus LP-MSCs, and greatest adipogenic potential for RP-MSCs. RP-MSCs also demonstrated the highest proliferation rate. Stem cell marker expression also varied by tissue source, with RP- and SC-MSCs, but not LP-MSCs, exhibiting upregulation of CD44, CD90, and CD105.

The above findings suggests that AD-MSC behavior, and therefore clinical results with autologous AD-MSC therapy in horses, may depend upon donor characteristics, as well as the specific source of fat.

**Note: Mechanisms of action**

MSC mechanisms of action are likely similar across species, but as described in previous white papers concerning dogs and cats, understanding in this area is incomplete. Two main mechanisms have been proposed: tissue replacement via differentiation of MSCs (e.g., MSCs become new cartilage), and effects on the local tissue microenvironment.[9-11] In this latter process, MSCs secrete a number of paracrine factors and participate in a variety of cell-to-cell interactions that suppress inflammation and facilitate tissue regeneration.[12] To learn more about this complex topic, see the 2016 review by Spees et al (*Stem Cell Res Ther.* 2016 Aug 31;7(1):125.).

## Osteoarthritis (OA)

Autologous equine MSCs have been evaluated in at least 8 controlled trials for treatment of experimentally induced and naturally occurring OA (**Table 1**).[9] A high safety margin is now well-supported, but efficacy results have been mixed. Due to variations in MSC type and dose, study protocol, and population characteristics, cross-trial comparisons are challenging, making firm conclusions elusive.[9,11] Even so, some studies have delivered promising results, potentially illuminating optimal treatment protocols.

Studies are described below. Because experimentally induced models of OA do not resemble naturally occurring OA (in terms of disease course, chronicity, innate healing, etc.) studies are sorted in kind, with experimentally induced models described first.[13]

### *Experimentally induced OA*

Five controlled trials have evaluated MSCs for treatment of experimentally induced OA.[14-17]



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The first study was conducted by Wilke and colleagues in 2007.[16] The investigators created bilateral, 15-mm, full-thickness cartilage lesions in the stifles of 6 horses (**Figure 1**). While the lesion in one knee was grafted with a self-polymerizing autologous fibrin vehicle containing 12 million BM-MSCs, the lesion in the other knee was grafted with the autologous fibrin alone. After 30 days, arthroscopy showed significantly improved healing in the treatment group (**Figure 2**); however, when horses were euthanized at 8 months, treatment and control lesions showed no significant differences in healing, and contained similar levels of proteoglycan and collagen type II (**Figure 3**).

*Figure 1: Chondral defect in A) treatment joint and B) control joint*

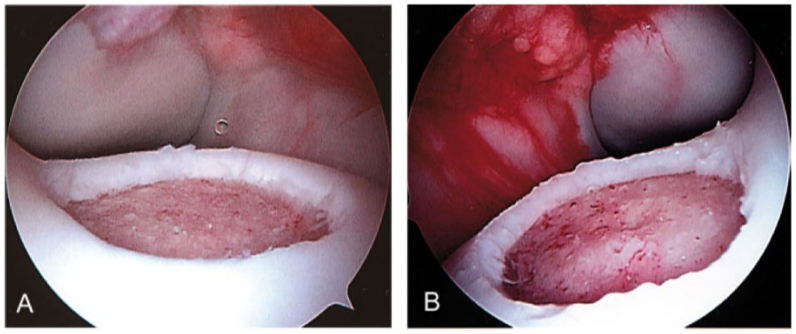



*Figure 2: Greater healing at 30 days in A) treatment joint vs B) control joint*

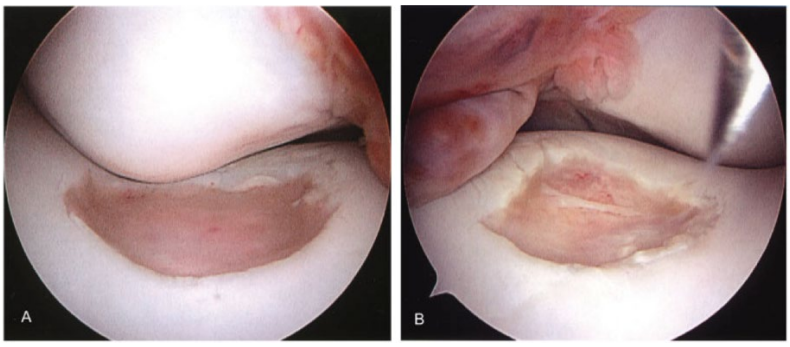
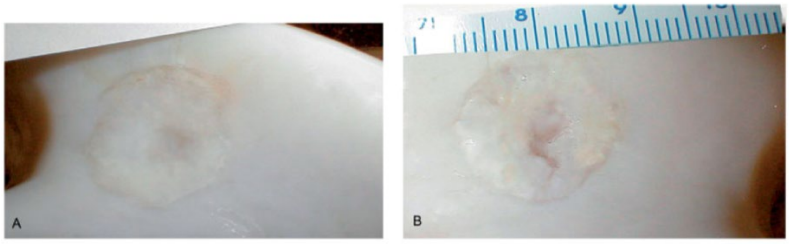



*Figure 3: Similar healing at 8 months in A) treatment joint and B) control joint*




The next study, conducted by Frisbie and colleagues in 2009, involved 24 healthy horses.[17] A full-thickness chondral defect (15 mm) was created in one middle carpal joint of each horse, while the



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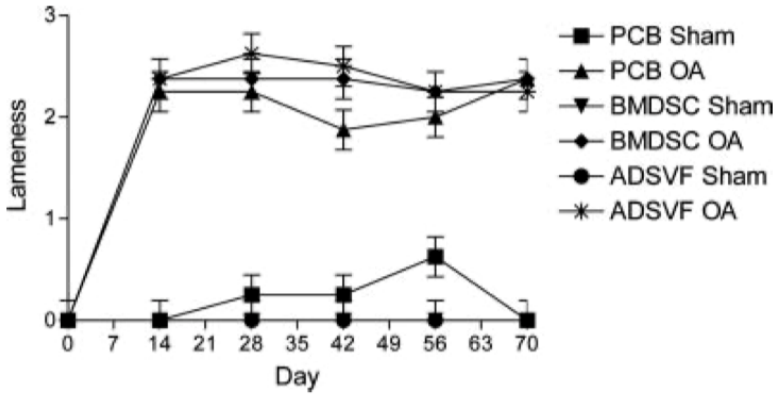
contralateral joint underwent sham procedure. Horses were then randomized in a 1:1:1 ratio to receive either an adipose-derived stromal vascular fraction (AD-SVF) containing 16.3 million cells, BM-MSCs at a dose of 10.5 million cells, or placebo (all intra-articular [IA]). Through day 70, no significant treatment effects were detected; all operated limbs exhibited similar lameness regardless of treatment group (**Figure 4**). Of note, however, horses treated with BM-MSCs had a significant reduction of prostaglandin E2 (PGE2) levels in both the treated joint and the sham joint, which suggests reduction in inflammation and possibly pain.

*Figure 4: Operated limbs exhibited similar lameness regardless of treatment type*




In a third study, published by McIlwraith and colleagues in 2011, ten healthy horses underwent arthroscopy to create 1 $cm^2$ defects in stifles bilaterally.[18] One month later, one joint was injected with 20 million BM-MSCs + hyaluronic acid, while the other joint received hyaluronic acid alone. From 4 months to 12 months, horses underwent strenuous exercise to simulate race training. Joint changes were evaluated bimonthly with radiography, via arthroscopy at 6 months, and via necropsy after euthanasia at 12 months. Although no clinically significant improvements were observed in BM-MSC-treated joints, 6-month arthroscopy and 12-month necropsy showed increased tissue repair firmness and a trend toward better tissue quality. In the BM-MSC joints, immunohistochemistry also revealed increased levels of aggrecan in repair tissue, supporting greater cartilage repair.

The two remaining OA model studies induced pathology by non-surgical means.

The first of these two studies (Mokbel, 2011) actually involved donkeys instead of horses, but the results offer clinical promise and some insight into MSC homing.[15] The investigators first induced OA by injecting Amphotericin-B bilaterally into the carpal joints of 27 donkeys. Donkeys were then divided into



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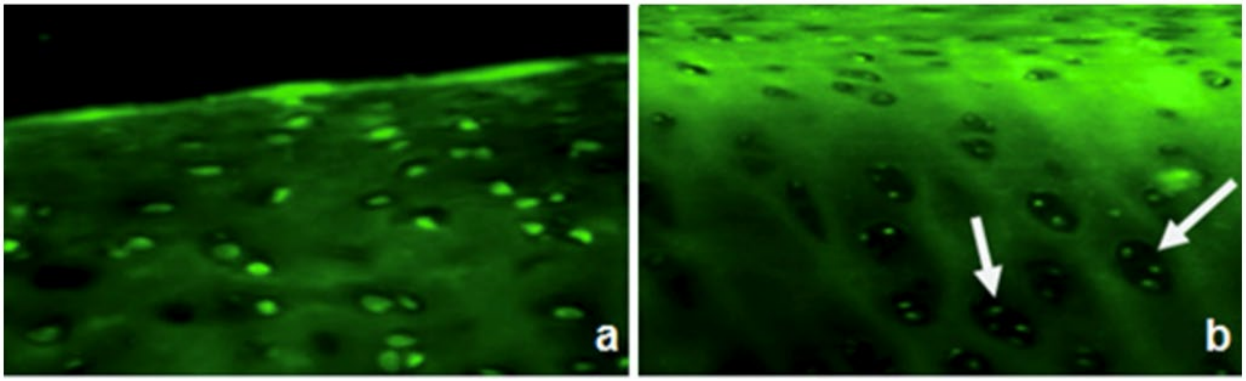
3 OA severity groups based on induction period until treatment (3 weeks, mild; 6 weeks, moderate; 9 weeks, severe). All subjects were injected with 5.4-6.9 million BM-MSCs + hyaluronic acid in their right carpal joint, while the left joints were injected with hyaluronic acid alone. MSCs were labeled with green fluorescent protein (GFP). Compared with hyaluronic acid alone, significant clinical and radiological improvements were seen in the treated joints across groups, with greatest improvement in joints treated soonest. Of note, fluorescent microscopy revealed green fluorescent protein-stained cells in the new and existing articular cartilage, suggesting participation in the regenerative process (**Figure 5**).

*Figure 5: GFP-stained cells in articular cartilage at a) 2 months and b) 6 months*




The final study using an OA model was conducted by Colbath and colleagues in 2020.[14] In both tarsocrural joints of 12 horses, recombinant interleukin-1β (rIL-1β) was injected to induce synovitis. In 8 horses, two doses of 10 million autologous BM-MSCs were then injected into one joint two weeks apart (week 1 and 3), while the other joint received an equal dose of allogeneic BM-MSCs (week 2 and 4). Compared with no treatment, neither treatment resulted in clinical or cytological improvement, and no differences were seen between autologous vs allogeneic treatments. While these results may suggest inefficacy of MSC therapy, the investigators noted that rIL-1β induced "an acute and severe synovitis," which may have been too severe to be addressed by MSC therapy. They also noted that this type of inflammation is not characteristic of OA.

### *Naturally occurring OA*

Two controlled trials have evaluated autologous equine MSCs in naturally occurring OA.[19,20]

In 2013, Nicpon and colleagues conducted a study involving 16 horses with naturally occurring OA in the hock (bone spavin).[19] Ten horses received an IA injection of 5 million AD-MSCs, 3 horses received an IA injection of betamethasone, and 3 horses received no treatment. At 1 month and 2 months, the betamethasone group showed improved lameness that waned thereafter. In contrast, improvements



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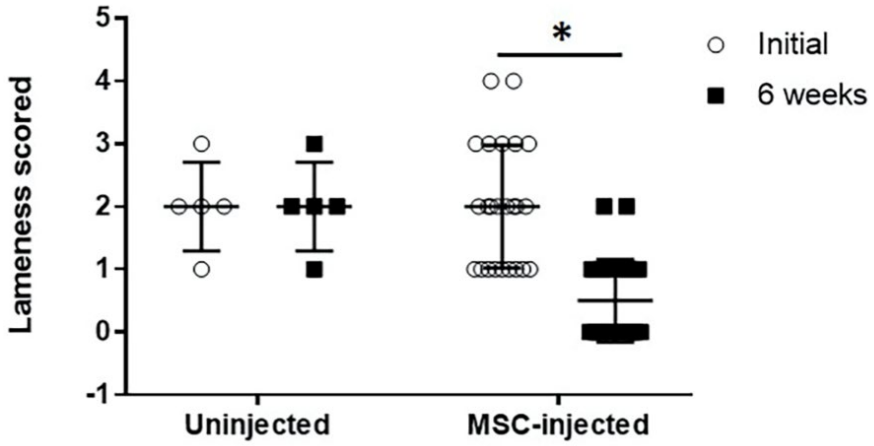
first appeared in the AD-MSC group at month 2, and lasted until month 6, suggesting later onset but also longer duration of action. At 3 months, both the betamethasone and AD-MSC group had reduced synovial inflammation. Again, this waned in the betamethasone group, while the AD-MSC group maintained synovial improvements until month 6. Untreated horses showed no improvement.

While all of the above studies involved administration of MSCs directly to the joint, Longhini and colleagues tested intravenous administration in 2019.[20] Twenty-four horses with naturally occurring OA in various joints were infused with 50 million peripheral blood-derived MSCs (PB-MSCs). After 6 weeks, treated horses showed significant improvements in lameness grade (**Figure 6**). Twenty-one out of 24 horses improved by at least one lameness grade, while 14 out of 24 horses had no lameness following treatment. Controls showed no improvements in lameness. Over 2 years after this initial study, 7 of the horses received 1-4 additional infusions of PB-MSCs without issue.

*Figure 6: Longhini 2019. Lameness scores in treated vs untreated horses*



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| Table 1. Controlled trials involving autologous mesenchymal stem cells for equine osteoarthritis | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **Lead author, year** | **Subject** | **Indication** | **n =** | **Cell type** | **Dose** | **Route** | **Protocol** | **Outcomes** |
| Wilke, 2007 | Horses | Surgically induced OA, stifle | 6 | BM-MSC | 12 million | Surgical | self-polymerizing autologous fibrin vehicle containing MSCs vs vehicle alone | Mesenchymal stem cell grafts improved the early healing response, but long-term assessment revealed repair tissue filled grafted and control lesions at 8 months, with no significant difference between stem cell-treated and control defects. Collagen type II and proteoglycan content in MSC-implanted and control defects were similar. |
| Frisbie, 2009 | Horses | Surgically induced OA, carpus | 24 | AD-SVF<br>BM-MSC | 16.3 million<br>10.5 million | IA | AD-SVF vs BM-MSC vs placebo | No significant treatment effects were demonstrated, with the exception of improvement in synovial fluid effusion PGE2 levels with bone marrow-derived mesenchymal stem cells when compared to placebo. A greater improvement was seen with BM-MSCs when compared to AD-SVF and placebo treatment |
| McIlwraith, 2011 | Horses | Surgically induced OA, stifle | 10 | BM-MSC | 20 million | IA | BM-MSCs + HA mg vs HA alone | No clinically significant improvements occurred; however, 6-month arthroscopy and 12-month necropsy confirmed a significant increase in tissue repair. Immunohistochemical analysis demonstrated more aggrecan levels in the repaired tissue treated with BM-MSC. |
| Mokbel, 2011 | Donkeys | Chemically induced OA, carpus | 27 | BM-MSC | 5.4-6.9 million | IA | BM-MSCs + HA vs HA alone | Clinical and radiological improvements in all treated groups, compared with the control groups. Fluorescence microscopy of sections of the cell-treated joints of all animals indicated that the GFP-transduced injected cells participated in the reparative process of the damaged articular surface and integrated within the existing articular cartilage. |
| Nicpon, 2013 | Horses | Natural OA, hock | 16 | AD-MSC | 5 million | IA | AD-MSCs vs betamethasone vs no treatment | Improvements in lameness and synovial fluid inflammation in both treatment groups. Improvement waned in betamethasone group after 60 days, but persisted in AD-MSC group until day 180. |
| Longhini, 2019 | Horses | Natural OA, various joints | 29 | PB-MSC | 50 million | IV | PB-MSCs (1-5 infusions) vs no treatment | Significant improvement in median lameness grade. Fourteen of 24 treated horses had no observable lameness after treatment. Control horses showed no clinical improvement. |
| Colbath, 2020 | Horses | rIL-1β-induced synovitis, hock | 12 | BM-MSC | 10 million | IA | auto BM-MSCs (2x) vs allo BM-MSCs (2x) vs no treatment | Neither autologous nor allogeneic treatments resulted in an improvement in clinical or cytological parameters. OA model questionable. |

OA = osteoarthritis; AD = adipose-derived; BM = bone marrow; PB = peripheral blood; MSC = mesenchymal stem cell; HA = hyaluronic acid; SVF = stromal vascular fraction; DMSO = dimethyl sulfoxide; GFP = green fluorescent protein; NCC = nucleated cell count; CRP = C-reactive protein; PGE2 = prostaglandin $E_2$; rIL-1β = recombinant interleukin 1 beta



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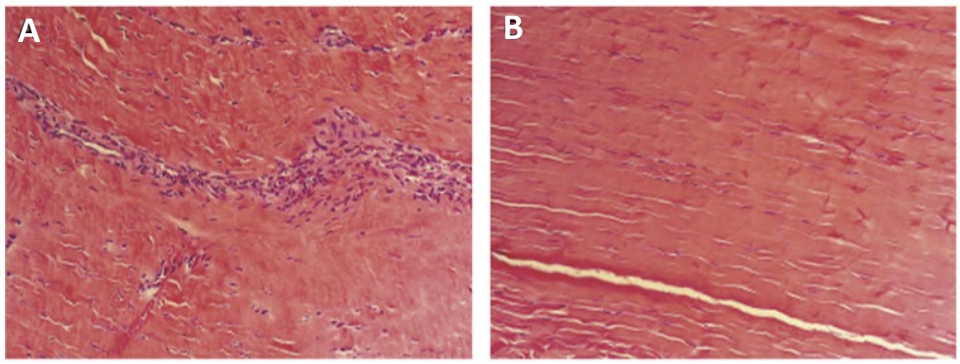
## Tendon and ligament injuries

Numerous studies have evaluated MSCs for tendon and ligament injuries in horses, most often involving injection of MSCs into the superficial digital flexor tendon (SDFT).[21] In general, efficacy results have been promising, and the vast majority of studies have reported a favorable safety profile.

Due to the volume of research conducted in this area, the summary below focuses on notable, controlled trials involving autologous MSCs (**Table 2**). Compared with controlled trials for OA, the following trials are more homogenous in terms of treatment dose (typically 10 million cells) and route of administration (directly into the lesion). Results are also more consistent, with most studies showing improvements on ultrasound and histopathology. Lacking, however, are signs of clinical significance. Between these 6 publications, significant clinical improvement is observed only once (Rivera, 2020), and in that instance, it is loosely defined, without close adherence to lameness scoring.

The first controlled MSC trial for tendon and ligament injuries was conducted by Carvalho and colleagues in 2011.[22] It involved 8 healthy horses that underwent collagenase injection to induce bilateral SDFT lesions. One lesion was treated with intra-lesional (IL) injection of 10 million AD-MSCs, while the other received no treatment. Although no improvements were seen clinically or on ultrasound through day 150, histopathology revealed improved tendon healing, including increased tendon organization, reduced inflammation, and increased type I collagen (**Figure 7**).

*Figure 7: A) Control joint shows more cellularity and less collagen fiber organization than B) treated joint*




Three years later, Carvalho and colleagues repeated this experiment in 8 other horses.[23] This time, however, 10 million AD-MSCs were given with platelet concentrate and compared with phosphate buffered saline (PBS). In contrast with the previous study, ultrasound revealed significant improvements



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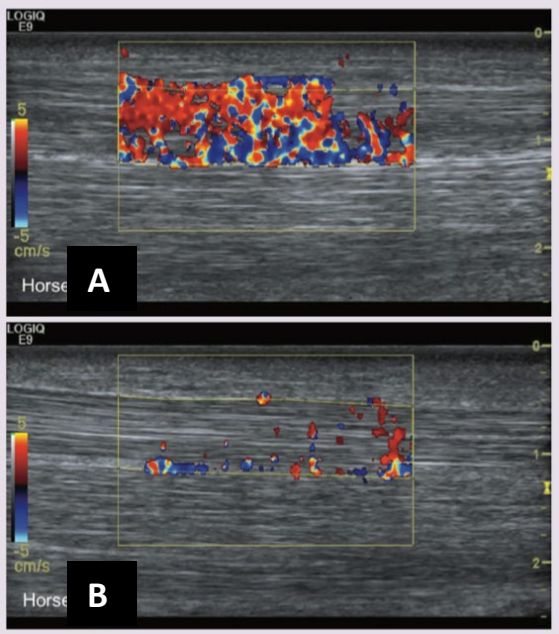
in MSC-treated tendons compared with controls (at week 16). Again, histopathology showed the benefit of MSCs, including reduced lesion progression, reduced inflammation, and increased fiber organization.

Two other studies induced bilateral SDFT lesions via a surgical method.

The first, by Conze and colleagues, involved 9 horses that were injected with 10 million AD-MSCs in one tendon and inactivated autologous serum in the other.[24] At 2 weeks, ultrasound showed increased blood flow in the treated tendon vs the control tendon (**Figure 8**). At 22 weeks, histopathology confirmed this finding with the presence of increased neovascularization.

*Figure 8: Color Doppler ultrasonography shows increased blood flow in A) treated tendon versus B) control*




The other study to use a surgically induced model of SDFT injury was conducted by Romero and colleagues in 2017.[25] Following bilateral tendon surgery, 12 horses received IL injections 20 million AD-MSCs, BM-MSCs, platelet-rich plasma (PRP), or lactated Ringer's solution (LRS) in contralateral limbs. Horses that received MSCs of either variety showed improved healing and tendon organization compared with controls. Compared with AD-MSCs, BM-MSCs were associated with earlier benefit, starting at week 6, and at week 45, higher expression of genes related to tissue regeneration.

The two remaining studies in this area of research involved naturally occurring SDFT injury.



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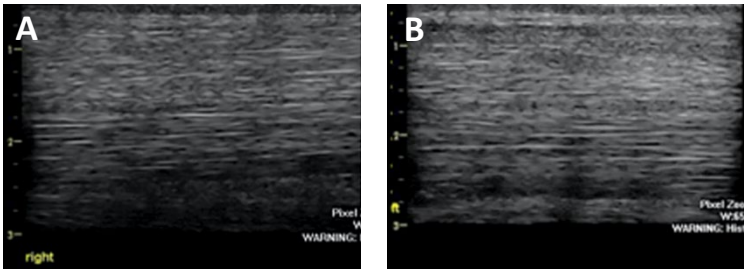
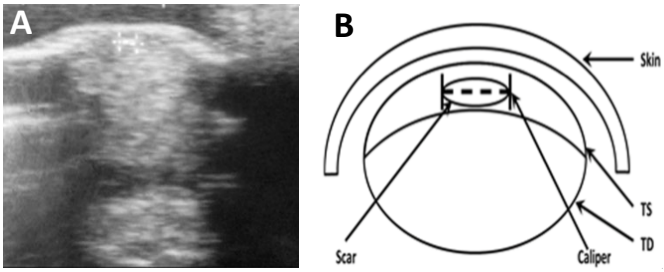
The first study, by Smith and colleagues, involved 12 horses that were randomized to receive injection with either 10 million BM-MSCs in bone marrow supernatant or PBS.[26] After 6 months, treated horses exhibited significantly improved tendon healing, based on reduced stiffness, reduced inflammatory infiltrate, increased tendon organization (**Figure 9**), and lower rate of reinjury.

*Figure 9: At six months, increased linear organization of A) treated tendon versus B) control*




The final and most recent study, by Rivera and colleagues, employed a novel method to quantify SDFT improvement.[27] Ten horses with naturally occurring, recurrent SDFT injury were randomized to receive either 600 thousand AD-MSCs or no treatment. Improvement was measured by SDFT "scar length" on ultrasound, allowing for objective comparison between groups (**Figure 10**). After 2 months, the treatment group exhibited significantly greater reductions in scar length. Compared with baseline scar length, after 4 months, treated horses had a mean scar length of 26.7%, versus 83.92% for controls ($P <$ .01). Clinical improvements, such as reduced pain on SDFT palpation and reduced lameness, were also observed in the treatment group during the first three weeks.

*Figure 10: A) Ultrasound image of SDFT scar measurement with calipers, and B) diagram of the ultrasound screen indicating the hyperechogenic regions of tendon of the deep digital flexor muscle (TD) and the scar (ellipse) on the tendon of the superficial digital flexor muscle (TS)*



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| Table 2. Controlled trials involving autologous mesenchymal stem cells for equine tendon and ligament injuries | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| **Lead author, year** | **Subject** | **Indication** | **Sample size** | **Cell type** | **Dose** | **Route** | **Protocol** | **Outcomes** |
| Carvalho, 2011 | Horses | Collagenase-induced SDFT lesion | 8 | AD-MSC | 10 million | IL | AD-MSCs vs no treatment | No clinically or ultrasonographically significant changes through day 150. But histopathology showed improved tendon healing, including increased tendon organization, reduced inflammation, and increased type I collagen. |
| Smith, 2013 | Horses | Natural SDFT injury | 12 | BM-MSC | 10 million | IL | BM-MSCs + BM supernatant vs PBS | Significantly improved tendon healing, including reduced stiffness, increased organization, and lower rate of reinjury. |
| Carvalho, 2013 | Horses | Collagenase-induced SDFT lesion | 8 | AD-MSC | 10 million | IL | AD-MSCs + PC vs PBS | Treated group had superior healing based on ultrasound and histopathology at 16 weeks. Treatment group had increased blood flow, reduced lesion progression, reduced inflammation, increased fiber organization. |
| Conze, 2014 | Horses | Surgically induced SDFT lesion | 9 | AD-MSC | 10 million | IL | AD-MSCs vs inactivated autologous serum | Increased neovascularization of treated tendons. |
| Romero, 2017 | Horses | Surgically induced SDFT lesion | 12 | AD-MSC<br>BM-MSC | 20 million | IL | AD-MSCs vs BM-MSCs vs PRP vs LRS | Treated horses showed improved healing and tendon organization compared with controls. Compared with AD-MSCs, BM-MSCs were associated with earliest benefit and higher expression of genes related to tissue regeneration at week 45. |
| Rivera, 2020 | Horses | Natural SDFT injury | 10 | AD-MSC | 600 thousand | IL | AD-MSCs vs no treatment | Significant reduction in SDFT scar length after 2 months. After 4 months, treated horses had a scar length 26.7% of baseline, compared with 83.92% for controls. |

AD = adipose-derived; BM = bone marrow; MSC = mesenchymal stem cell; IL = intralesional; PC = platelet concentrate;
PBS = phosphate buffered saline; LRS = lactated Ringer's solution; SDFT = superficial digital flexor tendon; PRP = platelet-rich plasma



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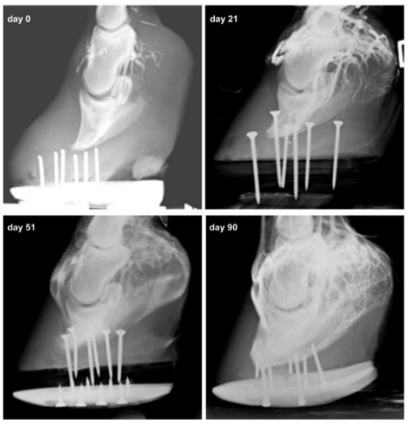
**Laminitis**

Compared with the body of evidence supporting MSCs for OA and tendon/ligament injuries in horses, laminitis research is scarce. To date, two studies have reported use of autogenous MSCs in cases of laminitis, although it is important to note that these treatments followed induction therapy with allogeneic MSCs. Neither study was controlled.

The first study, conducted by Dryden and colleagues, involved 30 horses with chronic laminitis.[28] Allogeneic umbilical cord blood-derived (UCB-MSCs) were used for initial treatment, followed by treatment "when possible," with autologous BM-MSCs. MSCS were administered via retrograde venous digital perfusion via the palmar/plantar digital vein. Horses received up to 4 treatments in 1-month intervals, each time with 20-30 million MSCs. Deep digital flexor tenotomy was also performed on at least 1 affected limb in at least 50% of the cases. In total, 21 patients (70%) had a successful outcome. All horses (100%) treated within 30 days of laminitis onset had a successful outcome, suggesting that earlier treatment may be more effective. No adverse events were reported.

A second uncontrolled study by Angelone and colleagues involved 9 horses with severe laminitis that had failed conventional therapies.[29] In 3 treatments given at 1-month intervals, 9 horses received 15 million AD-MSCs + PRP via the lateral or medial digital vein. Venograms showed progressive improvement in vascularization (**Figure 11**), plus improvements in hoof function and structure. All horses returned to "comfortable quality of life," and no adverse events were reported.

*Figure 11: Improvements in vascularization over a three-month period*



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The above two laminitis studies are promising, and the latter suggests that a combination of autologous MSCs + PRP may be most beneficial; however, interpreting the role of autologous MSCs is challenging, as results are confounded by initial treatment with allogeneic MSCs, and a lack of control groups. Further research in this area is needed.

### Exertional rhabdomyolysis

Equine studies evaluating MSC therapy for exertional rhabdomyolysis are lacking; however, preclinical research with a mouse model suggests potential. In the most-cited of these studies, by Geng and colleagues, rhabdomyolysis was induced by intramuscular injection of glycerol.[30] Six hours later, mice in the treatment group received an intravenous infusion of 1 million allogeneic BM-MSCs, while the control group received an equivalent volume of saline. Results showed that MSCs reduced renal function impairment and sever tubular injury. Histopathology also revealed increased CD206-positive M2 macrophage infiltration in kidneys, suggesting anti-inflammatory activity and transition toward tubule repair.

### Summary

MSCs have been under investigation for a variety of equine diseases for more than 20 years. During this time, investigators have focused on musculoskeletal conditions such as OA and tendon/ligament injury, although emerging research is expanding in scope to address a range of conditions, such as laminitis, peripheral nerve injury, laryngeal hemiplegia, and cervical vertebral malformation (CVM).[2]

Presently, a small body of evidence supports use of autologous MSCs for musculoskeletal conditions. While a number of studies have observed improvements via imaging and histology, clinical improvements are mixed. In controlled trials for OA, sample sizes have ranged from 6 to 29 individuals, while studies for SDFT injury have maxed out at 12 subjects. These small samples make it difficult to discern differences in treatments and controls, and therefore generate firm conclusions about treatment efficacy. A lack of repeated, long-term treatment — as may be expected when treating chronic conditions in the real world — is also generally lacking.

The 2019 study by Longhini and colleagues stands out among the studies involving autologous MSCs for OA.[20] Although the study was unblinded, and lameness exams are prone to subjectivity, treatment was



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associated with significant clinical improvements in lameness in 23 out of 24 treated horses, and 14 out of 24 treated horses had no lameness after treatment. Of note, the investigators used an intravenous dose of 50 million peripheral blood-derived, autogenous MSCs (versus intra-articular injections), and some horses safely received up to 5 infusions. As all 29 horses had naturally occurring OA involving various joints, this study may best approximates real-world practice. Ideally, a similar, albeit larger, randomized, blinded, placebo-controlled trial needs to be conducted.

For tendon/ligament injuries, no equivalent trial is available for autologous MSCs. Instead, multiple small trials have repeatedly demonstrated histological and ultrasound improvements in tendon healing associated with autologous MSCs. Apart from the study by Rivera and colleagues, which relied upon a poorly-defined measure of clinical success, no significant clinical improvements have been reported in this area. Further research is needed to explore effects on naturally occurring tendinopathy, as well as the impacts of repeated intra-lesional treatment, and intravenous delivery.



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