

Investigating the efficacy of amnion-derived compared with bone marrow–derived mesenchymal stromal cells in equine tendon and ligament injuries

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Abstract

Background aims. This is the first study to compare the treatment of horse tendon and ligament injuries with the use of mesenchymal stromal cells (MSCs) obtained from two different sources: amniotic membrane (AMSCs) and bone marrow (BM-MSCs). The objective was to prove the ability of AMSCs to exert beneficial effects *in vivo*. **Methods.** Five million allogeneic frozen-thawed AMSCs or autologous fresh BM-MSCs were injected intralesionally in horses belonging to group A (51 horses) and group B (44 horses). The interval lesion/implantation was of 6–15 days for the AMSCs and 16–35 days for the BM-MSCs. Healing was assessed clinically and ultrasonographically. Follow-up was monitored for 2 further years from return to full work. **Results.** No significant adverse effects after MSCs treatment were seen in any of the horses studied, independent of the type of stromal cell implanted. All animals belonging to group A resumed their activities between 4–5 months after treatment, whereas animals of group B resumed their activities after 4–12 months. The rate of re-injury in horses treated with AMSCs is lower (4.00%) compared with the average observed when horses were treated with BM-MSCs (23.08%). **Conclusions.** The possibility to inject allogeneic AMSCs in real time, before any ultrasonographic change occurs within the injured tendon and ligament, together with the higher plasticity and proliferative capacity of these cells compared with BM-MSCs, represents the main features of interest for this novel approach for the treatment of equine tendon diseases. An obvious active proliferative healing in the area injected with AMSCs makes these cells more effective than BM-MSCs.

Key Words: amniotic mesenchymal cells, bone marrow mesenchymal cells, equine, re-injury, tendon disease

Introduction

The use of stem cells in veterinary practice is focused on the treatment of orthopedic injuries, especially in the horse. Mesenchymal stem cells (MSCs) have been used experimentally and in a limited number of clinical cases for the surgical treatment of subchondral bone cysts, bone fracture repair (1) and cartilage repair (2,3). However, the most frequently described use in equine medicine is the treatment of sport-induced superficial digital flexor tendon (SDFT) and suspensory ligament (SL) lesions. These two structures are somewhat correlated from a biomechanical point of view, and they represent the most frequently affected soft tissues in competition horses.

Tendons and ligaments heal slowly after injury and rarely regain their original strength and elastic

qualities. Suboptimal healing, prolonged rehabilitation time and a high incidence of recurrence make degenerative injuries difficult to treat successfully. The prognosis for patients with tendon or joint injuries is often poor. Despite a short inflammatory phase after the injury incident, the healing process is dominated by fibroplasia and can be referred to repair rather than regeneration, with the formation of hypercellular scar tissue with poor extracellular matrix organization, in which stiffness is increased but elasticity is decreased compared with the original tendon tissue (4). Standard treatments are more conservative, and tendons require a long healing time, with high risk of re-injury during athletic performances.

Within recent decades, the focus of medical science shifted from repair to regeneration and thus

the research on tendonitis therapies has focused on regenerative medicine approaches to prospect normal, or close to normal, structure and function of an injured organ. Regenerative medicine, including stem cell-based tissue engineering, requires an exogenous cell source, and this approach comes from the knowledge that most tissues have a subpopulation or side population of tissue-specific progenitor cells used to replenish cells as the result of natural turnover and aid in postinjury repair (5). Nowadays, it is possible to choose among several sources of cells to use in regenerative medicine, and it is still not clear which one can be considered therapeutically optimal. MSCs isolated from bone marrow (BM) and fat hold the greatest hope for the effective tendon and ligament therapies; however, recently, other sources have been indicated as potentially useful for the same purpose, including extra-fetal stem cells, which can be easily recovered at birth and stored for future use (6,7), and embryonic or fetal embryonic-like stem cells (8,9). Although BM-MSCs represent the most widely investigated cells for application in veterinary regenerative medicine, it is important to emphasize that these cells have a limited potential in terms of *in vitro* proliferation capability (approximately 32 days for expansion from isolation to implantation). The delay of 2–4 weeks because of the expansion of MSCs before reaching the sufficient amount of cells needed for the treatment represents a limiting factor in the context of the use of autologous BM-MSCs (8). We previously demonstrated that implanted equine amnion mesenchymal stromal cells (AMSCs) are well tolerated by horses when allogeneic cells are transplanted (6); therefore, the purpose of the present study is to compare the efficacy in teno-desmic repair with the use of AMSCs and BM-MSCs.

Methods

Research animals

BM-MSCs used for this study were collected from adult horses of different ages and either sex. Allanto-amniotic membranes were obtained at term of normal pregnancies from different mares. All collections were performed according to approved animal care and use protocols of the institutional ethics committee and with the written consent of the owner.

Isolation and culture of AMSCs and BM-MSCs

Isolation of AMSCs was performed as reported by Lange-Consiglio *et al.* (6). Briefly, amnion fragments (approximately 12 g for an extension of 630 cm²) were incubated for 9 min at 37°C in phosphate-buffered saline (PBS) containing 2.4 U/mL dispase (Becton

Dickinson, Milan, Italy). After a resting period (5–10 min) at room temperature in high-glucose Dulbecco's modified Eagle's medium (HG-DMEM; EuroClone, Milan, Italy) supplemented with 10% heat-inactivated fetal bovine serum (FBS; Sigma Chemical, Milan, Italy) and 2 mmol/L L-glutamine (Sigma), the fragments were digested with 0.93 mg/mL collagenase type I (Sigma) and 20 mg/mL DNase (Roche, Mannheim, Germany) for approximately 3 h at 37°C. The amnion fragments were then removed, and mobilized cells were passed through a 100- μ m cell strainer (Sigma) before collection by centrifugation at 200g for 10 min. Before seeding the primary culture (P0), the obtained stromal cells were counted with the use of a Burkler chamber with trypan blue (Sigma) dye exclusion assay.

For maintenance of cultures, AMSCs were seeded at up to 1×10^5 cells/cm² in HG-DMEM supplemented with 10% FBS, 100 UI/mL penicillin-100 μ g/mL streptomycin (Euroclone), 0.25 μ g/mL amphotericin B (Euroclone), 2 mmol/L L-glutamine (Sigma) and 10 ng/mL of epidermal growth factor (Sigma) and incubated at 38.5°C in a humidified atmosphere (90%) with 5% CO₂.

As reported by Lange-Consiglio *et al.* (6), AMSCs demonstrated high plasticity, differentiating *in vitro* toward mesodermic and ectodermic lineages.

After expansion until passage 3 (P3), AMSCs were cryopreserved in HG-DMEM supplemented with 50% FBS and 10% dimethyl sulfoxide (Sigma) for a minimum of 6 months in liquid nitrogen. As reported by Lange-Consiglio *et al.* (6), equine AMSCs can also be frozen and recovered without loss of their functional integrity in terms of morphology, presence of specific stemness markers and differentiation ability, although the renewal capacity was slightly lower than that observed for freshly isolated cells (value average of doubling time of 1.16 days for fresh AMSCs and 1.88 days for cryo-preserved AMSCs).

As reported in a previous article of ours (6), immunocytochemical studies performed at passage 3 (P3) showed that AMSCs were positive for the expression of specific embryonic markers (TRA-1-60, SSEA-3, SSEA-4 and Oct-4). Meanwhile, reverse transcriptase–polymerase chain reaction performed at P1 and P5 showed expression of mesenchymal stem/stromal cell markers (CD29, CD105, CD44 and CD166) with negativity for CD34 at P1, although this marker began to be expressed by P5. The cells also expressed major histocompatibility complex (MHC)-I at both P1 and P5 but lacked MHC-II expression until P5.

For BM-MSCs, approximately 30 mL of BM was collected from the sternum of each injured horse by use of a Jamshidi biopsy needle (Biopsybell, Modena, Italy) (10 cm; 11 gauge) into syringes containing

Table I. Horse specifications and clinical aspect of injuries in group A animals treated with amniotic stromal cells.

Discipline	Number of horses (total = 51)	Age of horses (years)	Structure injured	Limb	Lameness		Edema		Thickening		Moderate lesions	Severe lesions
					Yes	No	Yes	No	Yes	No		
Show jumping	23	3–9	8 SL,	23 f	7	1	7	1	7	1	4	4
			13 SDFT		11	2	10	3	10	3	10	
			2 DDFT		1	1	1	1	1	1	2	0
Dressage	14	7–12	5 SL,	4 h,	3	2	3	2	3	2	2	3
			9 SDFT	10 f	6	3	6	3	6	3	7	2
Eventing	4	4–8	4 SDFT	4 f	1	3	1	3	1	3	3	1
Trotter	3	3–8	1 SL,	1 h,	0	1	0	1	0	1	1	0
Flat racing thoroughbreds	7	2–5	2 SDFT	2 f	1	1	1	1	1	1	1	1
			1 DDFT, 6 SDFT	7 f	1	0	1	0	1	0	1	0
					5	1	5	1	5	1	4	2

DDFT, deep digital flexor tendon; f, front; h, hind; SDFT, superficial digital flexor tendon; SL, suspensory ligament.

12 500 UI/mL heparin (Schwarz Pharma S.p.A., Milan, Italy). Samples were stored at 4°C and processed within few hours. BM-MSCs were isolated by centrifuging the BM samples at 400g for 10 min, removing the supernatant, collecting the buffy layer of mononuclear cells and washing twice in PBS. Before seeding the primary culture (P0), the obtained mononuclear cells were counted with the use of a Burkert chamber with trypan blue dye exclusion assay. The expansion was performed in the same conditions and medium of AMSCs. Doubling time study was performed as reported by Lange-Consiglio *et al.* (6) for each sample of BM-MSCs.

In a previous study (10), we characterized BM-MSCs by showing that they expressed the mesenchymal marker *CD105*, the adhesion molecules *CD29* and *CD44* and were negative for the hematopoietic marker *CD34*. Moreover, these cells were positive for *MHC-I* and negative for *MHC-II*. Immunocytochemical analysis revealed that BM-MSCs express Oct-4 and SSEA-4 and were weakly positive for TRA-1-60. In addition to this, BM-MSCs showed the capability to differentiate toward the osteogenic, adipogenic and chondrogenic lineages.

In vivo cell transplantation

Ninety-two privately owned sport horses were assessed clinically (presence or absence of lameness, pain, edema or swelling) and by ultrasound examination (Medison Sonovet Pico, Miami, FL, USA) for evidence of tendon disease by the same practitioner. All lesions were documented ultrasonographically in both transverse and longitudinal scans. The lesions were graded in six categories of quantitative assessment as described by Rantanen (11). To be included in the MSCs group, horses were required to have new, unilateral acute injuries and an ultrasound-detectable hypo-echoic lesion; thus far, only lesions graded from 4–6 were included. Later on, they will

be identified as mild (grade 4), moderate (grade 5) and severe (grade 6). For the purposes of this study, horses were randomly assigned into two groups. Fifty-one horses (group A) were treated with allogeneic frozen-thawed AMSCs and 44 (group B) with fresh autologous BM-MSCs. The population of each group was mixed, given the variability of the kind and severity of lesions and discipline for which each horse was used. An attempt was made to have similar proportions of horses from each discipline in each treatment group, irrespective of the severity of injury. The characteristics of these groups are reported in Tables I and II.

Permission was obtained from the institutional ethics committee and written consent from the owners, and, to evaluate their ability to exert beneficial effects *in vivo*, 5 million of the allogeneic frozen-thawed AMSCs or autologous fresh BM-MSCs at P3, both diluted in 1 mL of autologous plasma, were injected under ultrasonographic guidance into injured tendons of the horses belonging to group A and group B, respectively. The interval lesion/implantation was of 6–15 days for the AMSCs and 16–35 days for the BM-MSCs.

Hair was clipped with a No. 40 blade. Routine aseptic surgical preparation was performed with a chlorhexidine scrub and alcohol. A local anesthetic ring block (2% Mepivacaine) proximal to the injection site and mild sedation with Detomidine (Pfizer Italia, Latina, Italy) were the only restraints used. The SDFT lesions were injected through a longitudinal technique (preferred in these cases by the operator) with a 20-gauge, 70-mm needle, in a proximal-to-distal direction. All the other lesions were injected transversely with the use of a 21-gauge, 40-mm needle, inserted laterally. A sterile bandage was applied and maintained for a 48-h period in which horses were box-rested. The horses were then given hand-walking exercise for 15 min/day over 15 days and 30 min/day for another 15 days. No nonsteroidal

Table II. Horse specifications and clinical aspect of injuries in group B animals treated with bone marrow mesenchymal cells.

Discipline	Number of horses (total = 44)	Age of horses (years)	Structure injured	Limb	Lameness		Edema		Thickening		Moderate lesions	Severe lesions
					Yes	No	Yes	No	Yes	No		
Show jumping	14	4–9	4 SL,	14 f	2	2	2	2	2	2	2	2
			9 SDFT		4	5	4	5	4	5	3	6
			1 DDFT		0	1	0	1	0	1	1	0
Dressage	12	8–12	4 SL	6 h,	4	0	4	0	4	0	2	2
			8 SDFT	6 f	7	1	7	1	7	1	6	2
Eventing	5	4–8	5 SDFT	5 f	3	2	5	0	5	0	3	2
Trotter	5	4–7	2 SL,	2 h,	1	1	1	1	1	1	1	1
			3 SDFT	3 f	3	0	3	0	3	0	2	1
Flat racing thoroughbreds	8	2–6	1 DDFT,	8 f	1	0	1	0	1	0	1	0
			7 SDFT		4	3	4	3	4	3	4	3

DDFT, deep digital flexor tendon; f, front; h, hind; SDFT, superficial digital flexor tendon; SL, suspensory ligament.

anti-inflammatory drugs (NSAIDs) or antibiotics were given, on the basis of the aseptic technique. Horses were examined clinically and ultrasonographically at approximately monthly intervals after treatment during convalescence and rehabilitation up to 12–15 months. Follow-up information was obtained from re-training to another 2 years, or sooner if a re-injury had occurred.

Results are based on those horses that returned to their former athletic function.

Statistical analysis

The Pearson χ^2 test was used to assess differences in re-injury rate in horses treated with AMSCs or BM-MSCs. Values of $P < 0.05$ were considered significant.

Results

BM-MSC culture

Approximately 28 mL of bone marrow was collected from each horse, and approximately 240×10^6 of mononuclear cells were obtained from each sample. The samples showing the presence of MSCs in the first step were kept in culture for approximately 26 days, the time needed to reach confluence. On average, 8×10^6 MSCs were obtained from each sample, with a maximum of 25×10^6 and a minimum of 5.2×10^6 . The doubling time mean value for the BM-MSCs was 3.42 ± 0.22 days.

In vivo results

Tables I and II and Figure 1 report data related to the animal used in this study. To summarize, 90.20% (46/51) of the horses in group A and 81.82% (36/44) of the horses in group B showed tendon injury located in the forelimb. No significant

differences ($P = 0.37$) were observed between the two groups.

In group A, 66.67% (34/51) of patients and in group B, 72.73% (32/44) of patients were affected in the SDFT, without significant difference ($P = 0.66$) between the two groups. The other percentages were distributed among SL (27.45% and 22.73% in group A and group B, respectively) and deep digital flexor tendon (DDFT; 5.88% and 4.54% in group A and group B, respectively).

In group A, 70.59% (36/51) of horses and in group B, 65.91% (29/44) of horses were lame to the

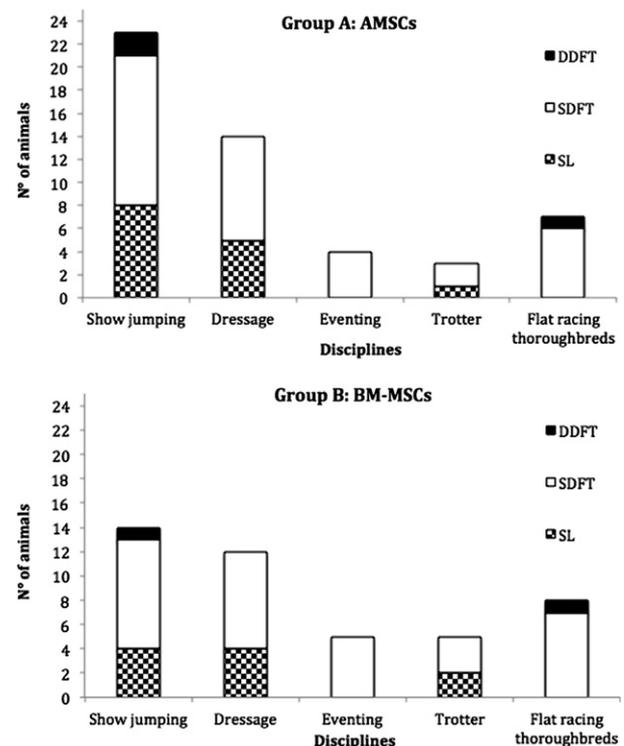


Figure 1. Horse and tendon/ligament distribution in the groups treated with amniotic and bone marrow-derived cells.

Table III. Incidence of recurrent injury in horses that returned to their former athletic function related to treatment group and discipline.

Discipline	Group A (total = 50)		Group B (total = 39)	
	Number of horses (%)	Structure injured	Number of horses (%)	Structure injured
Show jumping	0/22 (0.00)	–	3/12 (25.00)	1 moderate SL 2 severe SDFT
Dressage	2/14 (14.29)	2 Severe SDFT	4/9 (44.44)	1 moderate SDFT 2 severe SDFT 1 severe SL
Eventing	0/4 (0.00)	–	1/5 (20.00)	1 severe SDFT
Trotter	0/3 (0.00)	–	0/5 (0.00)	–
Flat racing thoroughbreds	0/7 (0.00)	–	1/8 (12.50)	1 severe SDFT

SDFT, superficial digital flexor tendon; SL, suspensory ligament.

clinical visit, without significant difference ($P = 0.66$) between the two groups.

In group A, 68.63% (35/51) of patients and in group B, 70.45% (31/44) of patients showed mild edema of the structures involved by the lesion. Generally, a moderate diffuse edema was always externally visible, without significant difference ($P = 1$) between the two groups.

In group A, 68.63% (35/51) of horses and in group B, 70.45% (31/44) of horses showed increased thickness of the affected area caused by inflammatory infiltration of the structure, without significant difference ($P = 1$) between the two groups.

In group A, the SDFT with severe lesion was 47.06% (16/34), whereas in group B, it was 43.75% (14/32), without significant difference ($P = 0.81$) between the two groups.

In both groups, the SL with severe lesion was 50% (7/14 and 5/10 for group A and group B, respectively), without significant difference ($P = 1$) between the two groups.

In both groups, the DDFT were characterized from moderate lesion (three in group A and two in group B).

On the basis of the data obtained for each of the features described, the two groups can be considered similar.

Follow-up information was available for all the horses treated. When AMSCs were allogeneically transplanted into horses from group A, all intralésional procedures were clearly well tolerated. No soreness or flares were noticed thereafter. Patients were able to walk and turn sharply soon after the implant, especially considering that no NSAIDs were administered. An early reduction in the degree of lameness, along with an appreciable volume reduction and decreased soreness on palpation, occurred in the treated horses just a few days after cell implantation. All animals belonging to the group A resumed their activity between 4–5 months after treatment except for one show jumping horse that died for reasons external to the study, as a result of an intestinal colic

3 months after the AMSC implantation. Two dressage horses were re-injured when returned to their former athletic level. These three horses had injury of the SDFT. Two years after cell transplantation, the average incidence of re-injury in horses treated with AMSCs was 4.00%, with the rate of 14.29% only in dressage horses (Table III and Figure 2).

The results obtained by injection of BM-MSCs showed that five of these horses (two show jumpers and three dressage) did not return to their former athletic function but were used as broodmares or were rested. The other animals of group B (39 horses) returned to work after a period varying between 4–12 months. After 2 years of cell transplantation, the incidence of re-injury ranged from 12.50% (1/8) to 44.44% (4/9) in different disciplines (Table III and Figure 2), with an average of 23.08%. Significant differences ($P = 0.0085$) are observed when these data are compared with those from re-injury after treatment with AMSCs.

No significant correlation was found between age and re-injuries ($r = 0.47$; $P = 0.2$), but there was a significant correlation ($r = 0.71$; $P = 0.02$) between disciplines and re-injuries, highlighting a trend for higher incidence of re-injury in flat racing thoroughbreds and in dressage horses than in other disciplines (Table IV). Comparing the ultrasound evolution of two different extremely severe injuries of the same tendon (acute partial rupture of SDFT) treated either with autologous BM-MSCs (case 1) or with heterologous AMSCs (case 2), in a similar time interval, it appears that in the second case, a more active sonographic evolution can be appreciated in a slightly shorter period, despite a more severe lesion, characterized by totally disrupted bundles and loss of longitudinal architecture (Figure 3).

Discussion

Intralesional injections of autologous BM-MSCs are widely used as a treatment in tendon diseases. This approach is time-consuming, being closely related

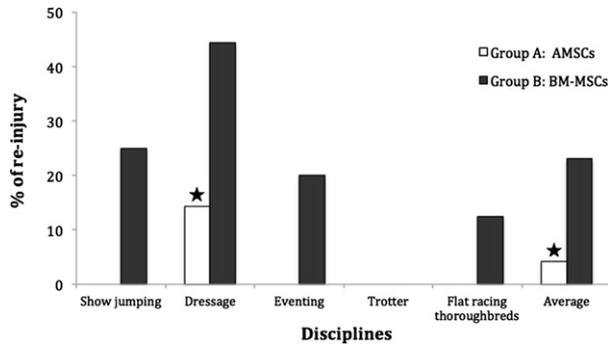


Figure 2. Rate of horses that showed recurrent injury after returning to their former athletic function related to treatment group and discipline. ★Statistically significant difference between groups.

to *in vitro* cell expansion that requires a 3-week period (12). In this time interval, ultrastructural changes occurring within the tendon represent limiting factors for an effective regeneration, predisposing the horse to re-injury. This study suggests that approximately 26 days are normally needed from the collection of BM from each injured horse to the cell injection into tendon lesions.

Because the goal of regenerative therapies is to restore normal structural architecture and biomechanical function to an injured tissue, it is important to implant the cells soon after the acute inflammatory phase and surely before intratendineous fibroblast migration and subsequent fibrous tissue formation (practically, this means implanting the tendon within 3 weeks after injury). For this reason, in some studies, the one-step BM-MSCs isolation is preferred, avoiding laboratory cell handling that could affect and limit BM-MSC proliferative and functional capacities after prolonged cultures, leading to a reduced repair potential (13). An increasing number of surgeons prefer the injection of marrow concentrate (14) because it allows the concentration of relatively few stem cells and the production of a cell pool with minimal cell manipulation and no risk of cell transformation during the growth *in vitro*. However, the precursors of bone and fat cells contained in BM may be

detrimental to the healing by triggering the formation of dystrophic mineralization or metaplasia at the site of inoculation.

Another source of MSCs commercially used in humans and animals is adipose tissue (15–18). The quick and successful recovery of adipose-derived MSCs through lipectomy (horses) (15) or collection of visceral fat during ovariectomy (dogs) (17), followed by enzymatic digestion, makes this tissue a promising source for clinical applications. Adipose tissue-derived MSCs appear to display a higher proliferation potential and lower senescence rate compared with MSCs from other sources (19–21). Several studies report a good differentiation potential into mesodermal tissue lineages (17,18), whereas other groups observed a limited chondrogenic and osteogenic potential in rat or horse adipose-derived MSCs (22,23). However, the collection of this tissue in the horse remains invasive. To overcome the limitations of BM and adipose tissue, extra-fetal tissues have been proposed as an attractive and alternative source of progenitor cells, which can open new perspectives in regenerative medicine. Extra-fetal sources of MSCs collected noninvasively are the umbilical cord (both, matrix and blood) and amnion. MSCs isolated from umbilical cord tissue represent a heterogeneous cell population, and therefore more rapidly proliferating cell populations must be isolated, for example, by sieving (24). To date, in horses there are no clinical approaches for the treatment of tendon diseases with the use of these cells, but Carrade *et al.* (25) injected autologous and allogeneic equine MSCs derived from either umbilical cord blood or tissue in noninjured horse joints to test the immunological properties of these cells. Their data suggest that there are no differences in the type or degree of inflammation elicited by self-related or non-self cells. Moreover, the inflammation was clinically mild and not associated with gait abnormalities or self-limiting, allowing us to conclude that with the mild to moderate self-limiting inflammation and the absence of a systemic immune response, placental-derived MSCs do not elicit any hyperacute or acute (MHC-mediated) immune response.

We have previously described the proliferative and differentiative potential of equine amnion-derived cells and the first therapeutic application in tendon injuries (6). In the present study, as in the previous one, the ultrasonographic evolution showed for tendon and ligament architecture is similar to that reported after injecting autologous BM-MSCs.

Comparing this innovative therapy with the conventional treatment with BM-MSCs, we observed that although the recovery time is very similar (only a minimum period of approximately 4 months is required in both groups), the rate of re-injury in

Table IV. Incidence of total re-injury related to discipline.

Discipline	Total number of horses	Number of horses with recurrent injuries	Percentage of re-injuries (%)
Show jumping	34	3	8.82
Dressage	23	6	26.09
Eventing	9	1	11.11
Trotter	8	0	0.00
Flat racing thoroughbreds	15	1	6.67

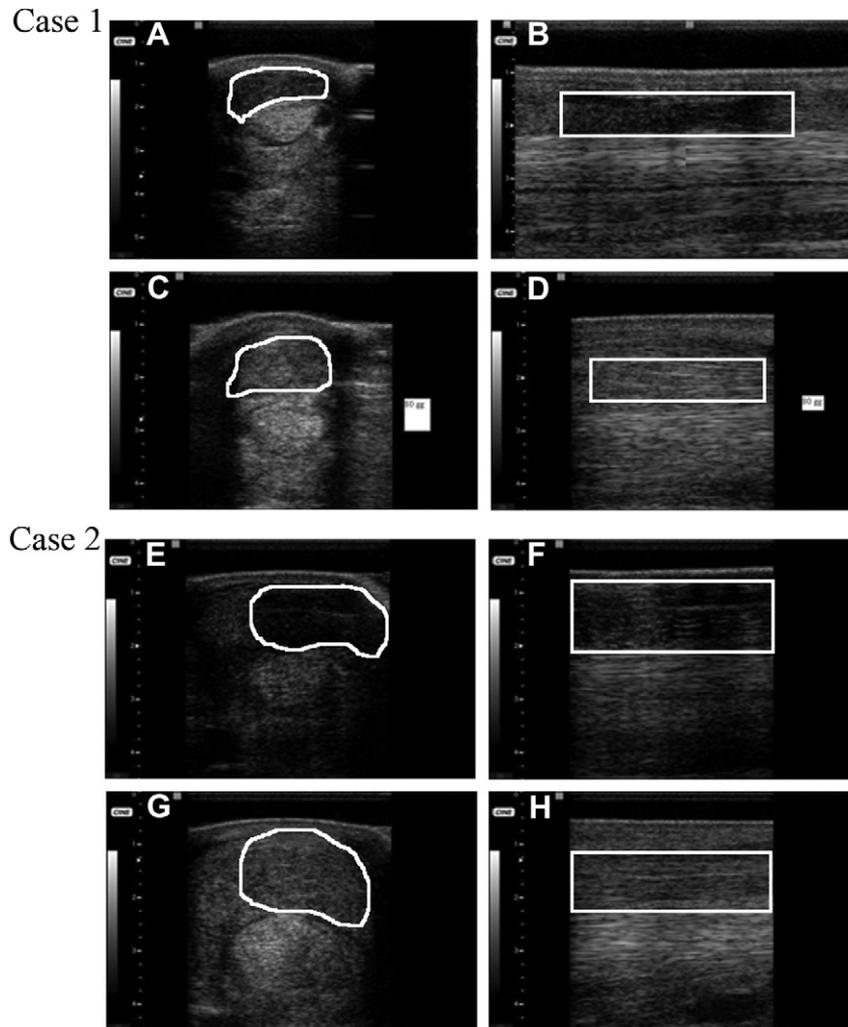


Figure 3. Case 1 was treated with BM-MSCs. Transverse and longitudinal ultrasound scans at cell injections, 25 days after injury (A, B) and evolution at 80 days (C, D). Case 2 was treated with AMSCs. Transverse and longitudinal ultrasound scans at cell injections, 10 days after injury (E, F) and evolution at 70 days (G, H). A more active sonographic evolution is appreciated despite a slightly shorter period and a more severe lesion in respect to case 1.

AMSC-treated horse is lower (4.00% versus 23.08% for AMSCs and BM-MSCs, respectively). The good result with AMSCs may be due to the opportunity to inject these cells in real time, before any ultrastructural change occurs within the injured tendon. Indeed, BM-MSCs require prolonged *in vitro* culture, limiting the time frame for implantation, and probably, in this situation, animals are prone to re-injury as the result of a nonoptimal regeneration. The regenerated tissue could be less elastic and therefore functionally inferior to normal or native tendon. The good time of implantation, together with the higher plasticity and proliferative capacity of the AMSCs compared with BM-MSCs, represents the main features of interest for this novel approach for the treatment of equine tendon diseases.

Unfortunately, it is still unclear whether the major contribution of the MSCs to the healing process is

to differentiate into tenocytes and thus produce extracellular matrix molecules, whether it is rather to supply growth factors and thus stimulate the residing cells within the tendon (4,26) or whether a combination of the two mechanisms occurs (27,28). Growth factors delivered with the stem cells may provide direction to the cells but may also stimulate native stem or other reparative cell populations. The timing of the implantation should be carefully considered as well. With the use of AMSCs, it could be hypothesized that the earlier implantation would offer greater benefits to the healing tendon by reducing inflammation, recruiting native stem cells and promoting production of collagen and other extracellular matrix proteins (29). Moreover, according to previous works (8,9) for equine embryonic stem cells and fetal-derived equine embryonic-like stem cells, it is possible to speculate that AMSCs, being most primitive

compared with adult cells, exhibit a higher level of engraftment in respect to BM-MSCs. As seen in the case of two different injuries of the same tendon treated either with autologous BM-MSCs (case 1) or with heterologous AMSCs (case 2), in a comparable time interval, it was evident that there was an obvious active proliferative healing in the area injected with AMSCs that was even more efficient than BM-MSCs. Moreover, it is important to underline that AMSCs used in these study were cryopreserved, thus demonstrating an even higher effectiveness in respect to fresh BM-MSCs. As previously reported (6) after freezing-thawing, cells were able to differentiate toward the same lineages tested for freshly isolated cells and expressed the same pluripotent and MSC-associated markers. Their proliferation rate was slightly lower than fresh cells but still higher compared with fresh BM-MSCs.

In our study, despite a heterogeneous case load consisting of different breeds, disciplines and injuries, mid-metacarpal SDFT lesions showed the higher incidence, in agreement with specific literature. Moreover, in both groups, the re-injuries mainly occurred in severe lesions of SDFT in dressage horses. Because the SDFT displays several similarities to the human Achilles tendon concerning anatomy, biomechanics and pathogenesis of diseases, the horse may represent an excellent model for human tendon studies. The strain-induced injury of this tendon is more likely to require a tissue regeneration process to achieve sufficient biomechanical strength and resistance to metacarpo-phalangeal hyperextension and energy-store function at high speed, identifying this peculiar tendon as a target indicator in regenerative medicine. Furthermore, the typical SDFT core lesion architecture provides an ideal cell injection site, acting as a natural scaffold (30), allowing intralesional injection of MSC suspension without any artificial support material; nevertheless, the effectiveness of an adequate ultrasound-guided technique, in minimizing further iatrogenic damages, cannot be underestimated (31). No correlation has been found in this mixed race and sport horse population between age and re-injury rate, as already highlighted by Dyson (32), even if not observed by Reardon *et al.* (33). High incidence of SDFT in dressage horses and associated high re-injury rate in this discipline have been found: SDFT tearing is a less common condition in this sport compared with flat racing, but a noticeable predisposition to re-injury has transpired in our data. This is a surprising and interesting finding that has, to our knowledge, only been underlined by Kold and Dyson (34). To our understanding, a different pathogenesis must be considered in these elite, often-middle aged, athletes. Supposedly, degenerative changes play a major role. The effect of aging and exercise on

biochemical composition of the whole tendon matrix is a known condition described by Smith (31), but an association with training cyclical fetlock and gait extension in high-quality and extravagant movers can reflect an individual acquired tendon structural degeneration, leading to weakness. A long-standing and prolonged metacarpo/tarsophalangeal extension phase during passage and even more in piaffe and canter pirouettes, together with prolonged training sessions, plays an intrinsic mechanical role. In accordance with general literature, the SL is the second most injured entity in high-level sport horses. A high re-injury rate is commonly described in SL desmitis, whatever the discipline or lesion severity. This is a high loading structure subjected to cycling overuse; no evidence-based data are available as for SDFT, but degenerative changes within this ligament must be considered.

Whether or not this biomechanical and/or biochemical mechanism leads to unsatisfactory healing in both SL and SDFT in this equine sport category will need further investigation, bringing us to a better understanding of the condition.

Despite the best results obtained with the AMSCs, this study shows a number of limitations. It is very difficult to demonstrate improved healing in treated animals because no control animals were included. Difficulties to determine a control population are correlated to the treatments given in the equine industry. Moreover, interpretation of the results can be misleading by the variety of athletic sports in which the horses were involved, the nature of injury, the severity of the initial injury and the age of the horse. In addition, statistical analysis is difficult to standardize, considering that the treatment of different equine tendons are highly variable. All these factors influence the prognosis. The optimal time of treatment and the optimal dose and route of administration of stem cells have not been determined yet for any stem cell type, lesion or animal species. Many authors agree with Richardson *et al.* (4), suggesting that the optimal timing of cell implantation is after the initial inflammatory phase but before fibrous tissue formation. It has been hypothesized that the presence of mature fibrous tissue within the tendon would make implantation more difficult and reduce the benefits of the stem cell therapy because of its persistence. Other issues are debated and concern the amount of stem cells needed to stimulate regeneration or the period of follow-up to allow a direct comparison. To date, there are no dose response-based studies: in literature, the range varies between $1-50 \times 10^6$ cells, depending on the extent of the lesion, and the follow-up ranges from 1 month to 3 years.

Further research studies will be required to answer these questions. Additionally, data provided in

this study demonstrate the absence of significant adverse effects induced by the MSCs treatment in any of the horses studied. The clinical and ultrasonographic evaluation of tendons did not reveal evidence of inappropriate tissue or tumor formation. Clinical outcomes after MSC transplantation were favorable; however, the significant lower rate of re-injuries observed after AMSC injection let us suppose that the treatment with amnion-derived stromal cells is more efficacious compared with BM-MSCs implantation.

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