Allogenic Mesenchymal Stem Cells as a Treatment for Equine Degenerative Joint Disease: A Pilot Study

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Abstract: Cell-based therapies, such as treatments with mesenchymal stem cells (MSCs) and platelet-rich plasma (PRP) are thought to have beneficial effects on the clinical outcome of orthopedic injuries, but very few animal studies with large sample size are published so far. Therefore, the aim of this study was to assess the safety and report the clinical outcome of allogenic, immature or chondrogenic induced MSCs in combination with PRP for the treatment of degenerative joint disease (DJD) in 165 horses.

MSCs and PRP were isolated from a 6-year-old donor horse and transplanted either in their native state or after chondrogenic induction in combination with PRP into degenerated stifle (n=30), fetlock (n=58), pastern (n=34) and coffin (n=43) joints. Safety was assessed by means of clinical evaluation and the outcome was defined as failure to return to work (score 0), rehabilitation (score 1), return to work (score 2) and return to previous level (score 3), shortly (6 weeks) after treatment or at 18 weeks for the patients that returned for long-term follow-up (n=91).

No adverse effects were noticed, except for three patients who showed a moderate flare reaction within one week after treatment of the fetlock joint without long-term effects (1.8% of 165 horses). Already after 6 weeks, 45% (native MSCs) and 60% (chondrogenic induced MSCs) of the treated patients returned to work ($\rightarrow$ score 2+3) and the beneficial effects of the treatment further increased after 18 weeks (78% for native MSCs and 86% for chondrogenic induced MSCs). With the odds ratio of 1.47 for short-term and 1.24 for long-term, higher average scores (but statistically not significant) could be noticed using chondrogenic induced MSCs as compared to native MSCs. For all three lower limb joints a higher percentage of the treated patients returned to work after chondrogenic induced MSC treatment, whereas the opposite trend could be noticed for stifle joints. Nevertheless, more protracted follow-up data should confirm the sustainability of these joints.

Keywords: Allogenic, arthrosis, chondrogenesis, horse, stem cells.

INTRODUCTION

Degenerative joint disease (DJD) is a major cause of reduced athletic function and retirement in equine performers [1-3]. Conventional therapies only aim for alleviating the symptoms or enhancing clinical recovery for a short period of time, whereas regenerative medicine aims for long-term clinical improvement and pain relief of the affected joints [4, 5]. Equine (n=33) [6] as well as human (n=12+28) [7, 8] studies confirm a clinical improvement of patients with knee injuries after intra-articular autologous mesenchymal stem cell (MSC) injection, where others failed to demonstrate comparable clinical effects in mid carpal joints in horses [9]. Furthermore, due to many interspecies similarities, the horse can be considered as a valuable animal model to evaluate human orthopedic therapies [10]. For all the aforementioned reasons, MSC studies in equine joint pathologies are of therapeutic interest to the scientific community.

So far, the use of stem cell therapies in the joint is hampered by the occurrence of flare reactions after intra-articular injection, which has been described to occur in approximately 9% of the treated equine patients with autologous MSCs [6]. The main issue with inflamed areas is that interleukin (IL)-1β significantly increases the expression of pro-inflammatory cytokines, such as tumor necrosis factor (TNF)-α, IL-1β, IL-6 and IL-8, and therefore, modulate the transcription of paracrine signaling molecules in MSCs [11]. Indeed, the micro-environment - or niche - in degenerated cartilage might not provide the correct signals for MSC differentiation or alternatively, may even negatively influence their regenerative capacities. Therefore, a priori chondrogenic induction of MSCs may improve the clinical outcome. It has been reported previously that *in vitro* differentiation enhances the clinical and histological outcome of patients with osteoarthritis [12]. Importantly, short-term induction processes do not lead to increased expression of major histocompatibility complex (MHC) type I and II [5], which are considered to be major rejection proteins.

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In the present study, peripheral blood (PB) from one donor horse was used to isolate MSCs and platelet-rich plasma (PRP) for combined use, which leads to a more uniform and standardized treatment comparison evaluation. Furthermore, it has been confirmed that PRP increases the cell viability of chondrocytes, enhances the migration and chondrogenesis of MSCs and improves the clinical outcome of MSC therapy in osteoarthritic joints [5, 13]. In this regard, previous studies describe the safe use of allogenic PRP and MSCs for the treatment of several musculoskeletal injuries in horses and even humans [14-22]. As it has been reported that the clinical effect of PRP alone is significantly inferior to the MSC-PRP combination [5], all patients in the present study underwent combination treatment.

So far only few studies with a limited number of animals report the clinical application of allogenic MSCs and PRP for the treatment of degenerative joint disease (DJD) in horses. Furthermore, there is no data available on the frequency of adverse events when using these cell-based products in an allogenic set-up. Therefore, the aim of this study was to evaluate the clinical effects of immature MSCs in combination with PRP versus chondrogenic induced MSCs plus PRP in a large cohort.

Patients with naturally occurring DJD of stifle, fetlock, pastern and coffin joints were included in the study and assigned to one of the combination treatments. Short-and long-term follow-up consisted of a clinical evaluation and scores were given for failure of return to work (0), rehabilitation (1), return to work (2) and return to previous level (3).

MATERIALS AND METHODS

This study was carried out in accordance with the recommendations of the Animal Welfare Department of the Belgian Federal Public Service of Health. The used protocols were approved by the Committee on the Ethics of Animal Experiments (EC_2012_001 and EC_2013_001) of Global Stem cell Technology (Permit Number: LA1700607). All injections were performed after sedating the horses, and all efforts were made to minimize suffering.

Isolation and Chondrogenic Induction of Mesenchymal Stem Cells (MSCs)

In total, 50 ml of blood was collected in sterile EDTA tubes from the *vena jugularis* of a 6-year-old donor gelding, which was tested for different transmittable diseases at Böse laboratory (Harsum, Germany), as previously reported by our group [16]. Approval of the ethical committee was obtained (EC_2012_001). In order to isolate mesenchymal stem cells (MSCs), the blood sample was centrifuged at 1000 G for 20 minutes and the interphase was collected and diluted 1:2 in phosphate buffered saline (PBS) 1x. Afterwards, this suspension was gently layered on an equal amount of Percoll$^®$ density gradient (GE Healthcare).

After subsequent washing of the buffy coat, $20x10^6$ peripheral blood mononuclear cells (PBMCs) were seeded per T_{75} flask in 3 flasks and expanded in medium consisting of low glucose (LG) DMEM (Gibco), 20% foetal calf serum (FCS, Gibco) and 1% antibiotics-antimycotics (AB/AM, Sigma). The medium was refreshed twice a week and the cells were maintained at 37°C and 5% CO_2. At 60% conflu-

ency, the cells were trypsinized with 0.25% trypsin-EDTA and subcultured until passage 3, at which time cells were characterized as previously described [23] before seeding them at $6.7x10^3$ MSCs/cm² in T_{75} flasks for expansion, or chondrogenic induction in a two-dimensional culture system. Chondrogenic induction medium consisted of DMEM LG, 20% FCS, 1% AB/AM and cartilage-specific growth factors, as previously reported [5]. At the next confluency, native and chondrogenic induced cells were trypsinized, resuspended in 1 ml of DMEM LG with 10% of dimethyl sulfoxide (DMSO, Sigma) and frozen before being shipped on dry-ice for clinical application. Only for stifle injections, a double dose of 2 confluent T_{75} flasks was used for each patient.

Preparation of Platelet-Rich Plasma (PRP)

In 6 production series, 200 ml of peripheral blood was taken per citrate phosphate dextrose adenine-1 (CPDA-1) single blood bag (Terumo$^®$) for platelet-rich plasma (PRP) preparation. From the same donor horse (gelding) as for MSC isolation, 6x30 samples of 1ml PRP were prepared as previously described by our group [14, 16]. Each sample contained approximately $130x10^6$ platelets and was frozen and stored at -80°C before clinical application.

Patient Inclusion Criteria

For this study, 165 acceptor horses (female and male) from two veterinary practices were selected based on their injuries. To be included in this study, clinical lameness had to be present in at least a mild form for more than 2 months. Moreover, the observed locomotory disorder had to be attributable to stifle, fetlock (metacarpophalangeal or metatarsophalangeal), coffin or pastern joint osteoarthritis. In this regard, the source of the lameness was confirmed by both local anaesthesia and a positive flexion test for all the patients. Wherever applicable, the lameness was exacerbated by a flexion test of the joint, and was abolished by intra-articular administration of a local anaesthetic solution. In the present study, 0.5% Mepivacaine Hydrochloride solution was used, and horses were evaluated 10 minutes after injection. Furthermore, for horses to be included, radiographic (X-ray) or computer-tomographic (CT) signs of osteoarthritis of the joint had to be noticeable in the form of osteophytes and/or cartilage defects. Untreated or placebo animals could not be included in the present study, since only owner horses with naturally occurring DJD were used.

Injecting Mesenchymal Stem Cells (MSCs) and Monitoring of Adverse Reactions

For each horse, the intra-articular injection was performed at least 24 hours after local anaesthesia, since it has been reported that exposure of MSCs to high concentrations of anaesthetics negatively influences cell viability [24]. In addition, 0.04 mg/kg detomidine (Domosedan$^®$) and 0.1 mg/ml butorphanol (Turbogesic$^®$) were administered intravenously, for their sedative and analgesic effects, respectively. Horses were assigned by the treating veterinarians to native MSCs and PRP (Combination 1), or chondrogenic-induced MSCs and PRP (Combination 2) treatment. After thawing, both MSCs and PRP were aspirated in the same syringe (for combination groups) and administered intra-articulary. After the treatment, the horses were closely moni-

tored for 1 week by means of a daily examination of the injected joint and by observing the occurrence of adverse effects or hypersensitivity reactions (wheal formation, sweating, increased respiratory rate or even fever). Subsequently, the joints were evaluated at an average of 6 weeks (short-term) and 18 weeks (long-term) post injection through clinical evaluation by the treating veterinarians for all horses.

Outcome Scoring System

In order to clinically evaluate both treatments, the following parameters were graded by the same veterinarians at a short-term and long-term: failure to return to work = score 0, rehabilitation = score 1, return to work = score 2 and return to previous level = score 3. All the horses in this study showed initially at least a mild lameness, mild to moderate response to flexion test and moderate to severe joint effusion. As a result, all horses had very similar initial clinical signs.

Statistical Analysis

The two treatments were compared for the clinical score (4 ordered categories) at both time points using the multinomial model with cumulative logits and introducing indication and induction as categorical fixed effects. The likelihood ratio test at the 5% significance level was used to test whether the odds ratio differs significantly from 1 (SAS Version 6.3.).

RESULTS

Isolation and Chondrogenic Induction of Mesenchymal Stem Cells (MSCs)

The first spindle shaped cells were noticed after 17 days in culture and were isolated at 21 days at approximately 60% confluency. The characterization experiments revealed the same MSC properties as previously described [5, 23]. To confirm chondrogenic induction, cell morphology and glycosaminoglycan production was analysed by means of histological (Safranin O and Alcian Blue) and immunohistochemical stainings (collagen type II) (data not shown). Briefly, a moderate increase of all the aforementioned stainings could be noticed in the two-dimensional culture system. In addition, the increase in typical cartilage genes (aggrecan, collagen type II and cartilage oligomeric matrix protein) indicated chondrogenesis as well (data not shown).

Adverse Reactions

After the intra-articular treatment, a daily examination of the injected joint was performed during 1 week for the occurrence of local adverse effects, such as swelling, warmth, hypersensitivity or even non-weight bearing lameness. Subsequently, the joints were evaluated at approximately 6 weeks (short-term) and 18 weeks (long-term) post injection through clinical evaluation by the treating veterinarians for all horses. In the present study, no adverse effects were noticed in both treatment groups for stifle, coffin and pastern joints. However, in the largest group of horses (n=41), being the chondrogenic induced MSC-treated fetlocks, three horses showed a moderate flare reaction within 1 week after the treatment. For these patients, non-steroidal anti-

inflammatory drugs (NSAIDs) were administered during 3 days (Phenylbutazone: day 1 = 3g, day 2 = 2g and day 3 = 1g) resulting in a normalisation of the joint. Although 2 out of these 3 horses returned to work at the short-term follow-up, the administration of the NSAIDs might have influenced these observations. Furthermore, the occurrence of possible general adverse effects or hypersensitivity reactions (wheal formation, sweating, strong respirations or even fever) was monitored as well. All the treated patients were comfortable during the entire follow-up period and no general adverse effects could be noticed by the veterinarian, owner or caretaker.

Outcome Scoring Results

In the present study, 165 equine patients were intra-articulary injected in their injured joint. The largest group consisted of fetlock joints (n=58), followed by coffin joints (n=43), pastern joints (n=34) and stifle joints (n=30) (Table **1**). No significant differences were found between the induced and the native MSCs, with the odds ratio for the short term being 1.47 (p=0.266) and for the long term being 1.24 (p=0.641), thus resulting on average in higher observed scores for induction as compared to native MSCs.

For the stifle joints that were treated with native MSCs, no horses failed to return to work at both observation points, whereas 7% of the chondrogenic induced MSC-treated patients failed to return to work at the long-term (Table **1**, Fig. **1**). For fetlock joints on the other hand, still 12% (short-term) and 11% (long-term) of the horses failed to return to work after native MSC-treatment, whereas none of the patients failed to return to work after chondrogenic induced MSC-treatment at both time points (Table **1**, Fig. **1**). The same trend could be noticed for coffin joints with 8% (short-term) and 17% (long-term) in the native MSC-treated patients versus 0% (short- and long-term) in the chondrogenic induced MSC-treated group (Table **1**, Fig. **1**). For the pastern, 9% (short-term) and even 40% (long-term) of the native MSC-treated horses failed to return to work, whereas 0% failed to return to work at the long-term observation point after chondrogenic induced MSC-treatment (Table **1**, Fig. **1**). Overall, the average short-term and long-term follow-up demonstrated less failure to return to work in the chondrogenic induced MSC-treated groups (Fig. **1**).

When looking at the number of horses with stifle DJD that are working (i.e. return to work + return to previous level), 63% of the patients treated with native MSCs were working at the short-term and 80% at the long-term, whereas 55% of the horses treated with chondrogenic induced MSCs were working at the short-term and 73% at the long-term (Fig. **1**). At the short-term, a higher percentage of the horses treated in the fetlock (41% vs 73%), coffin (38% vs 53%), as well as the pastern (36% vs 61%) joints were working after chondrogenic induced MSC treatment versus native MSCs (Fig. **1**). The long-term time points displayed similar numbers that are working after native MSC- or chondrogenic induced MSC-treatment of the fetlock (89% vs 86%) as well as the coffin (83% vs 82%) joint (Fig. **1**). For the pastern joint, a higher percentage of the horses was working after chondrogenic induced MSC-treatment (100%) in comparison to the native MSC-treatment (60%) (Fig. **1**). Looking at the average scores per group, a similar observation could be

Table 1.   **Percentage of treated patients corresponding with their short- (n_1) and long-term (n_2) scores (from 0-3) after treatment with allogenic platelet-rich plasma (PRP) in combination with native mesenchymal stem cells (MSCs) or chondrogenic induced MSCs.**

| Joint	Treatment	0		1		2		3	
		Short	Long	Short	Long	Short	Long	Short	Long
Stifle	Native (n_1=8, n_2=5)	0.0	0.0	37.5	20.0	37.5	60.0	25.0	20.0
	Induced (n_1=22, n_2=15)	0.0	6.7	45.5	20.0	45.5	33.3	9.1	40.0
Fetlock	Native (n_1=17, n_2=9)	11.8	11.1	47.1	0.0	5.9	44.4	35.3	44.4
	Induced (n_1=41, n_2=22)	0.0	0.0	26.8	13.6	61.0	45.5	12.2	40.9
Coffin	Native (n_1=13, n_2=6)	7.7	16.7	53.8	0.0	15.4	50.0	23.1	33.3
	Induced (n_1=30, n_2=17)	0.0	0.0	46.7	17.6	46.7	52.9	6.7	29.4
Pastern	Native (n_1=11, n_2=5)	9.1	40.0	54.5	0.0	18.2	20.0	18.2	40.0
	Induced (n_1=23, n_2=12)	4.3	0.0	34.8	0.0	43.5	66.7	17.4	33.3
Total	Native + Induced (n_1=165, n_2=91)	3.0	5.5	40.6	11.0	40.6	47.3	15.8	36.3
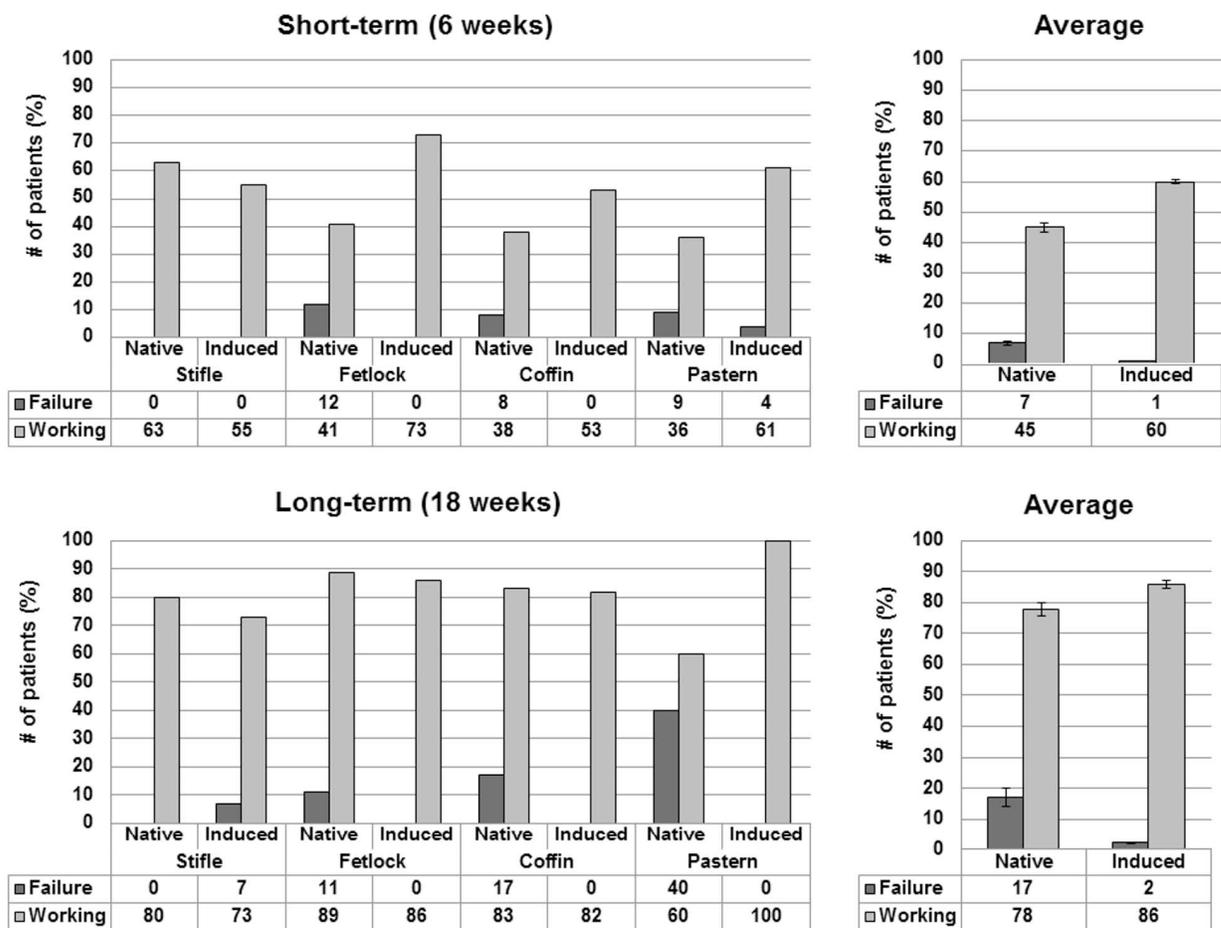
Fig. (1). Short-term (6 weeks) and long-term (18 weeks) functional outcomes: failure to return to work (score 0) versus horses in work (= return to work (score 2) + return to previous level (score 3)). Equine patients with naturally occurring degenerative joint disease (DJD) in stifle, fetlock, coffin or pastern joint were treated with platelet-rich plasma (PRP) in combination with native mesenchymal stem cells (MSCs) or chondrogenic induced MSCs. Values are given as the percentage of patients in each functional outcome group per joint or the average per treatment ± SEM.

made at short-term, however, only small differences in average scores could be noticed between both treatments at long-term (Fig. **2**).
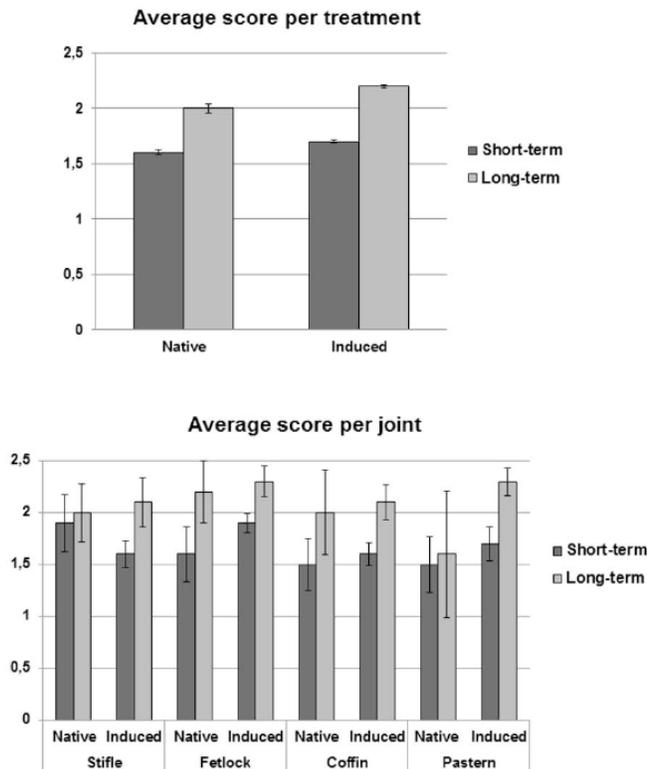

Fig. (2). Short-term (6 weeks) and long-term (18 weeks) average scores of equine patients with naturally occurring degenerative joint disease (DJD) in stifle, fetlock, coffin or pastern joint treated with platelet-rich plasma (PRP) in combination with native mesenchymal stem cells (MSCs) or chondrogenic induced MSCs. Values are given as the average score per joint or per treatment ± SEM.

DISCUSSION

The isolated cells in the present study showed the characteristics of mesenchymal stem cells (MSCs) according to the proposed guidelines by Dominici in 2006 [25] and were chondrogenically induced in a two-dimensional culture system as previously reported by our group [5]. The samples were frozen in this study to add to the product shelf life and to avoid a decline in cell number and viability during transport [26]. This study investigated whether *in vitro* chondrogenic induction of equine peripheral blood (PB)-derived MSCs enhances the outcome of degenerative joint disease (DJD) and whether the outcome would be joint-specific (stifle, fetlock, coffin and pastern joint).

In the present study, PRP was applied to both MSC treatments. Usage of PRP was anticipated to improve the clinical outcome as it has been reported that PRP enhances MSC proliferation and chondrogenic differentiation [27]. Moreover, it has been described that in comparison to PRP treatment alone, both combination therapies significantly enhance early and late clinical scores of horses with naturally occurring fetlock DJD [5]. Although the additional value of PRP to MSCs is being discussed in explant cultures [28], the aforementioned study suggested a synergistic clinical effect of PRP and MSCs in horse DJD. As we utilized allogenic PRP and MSCs from one donor in this study, the experimental model was more standardized for all patients, allowing a more accurate comparison between the different treatment groups. Several independent researchers report no increase in immune response after a single injection of allogenic MSCs in mammal joints [18, 29]. Also in this study, there were no clinical indications of severe immune response compromising the clinical outcome after allogenic MSC or PRP treatment. However, in three horses with fetlock DJD (n=41) that were treated with chondrogenic induced MSCs plus PRP, a moderate flare reaction without long-term effects could be observed within a week after the injection. Nonetheless, flare reactions have been previously described after intra-articular injection of native autologous bone marrow-derived MSCs as well [6]. In fact, Ferris *et al.* noticed a flare in three out of 33 (9%) injured equine stifle joints, whereas in the present study no flare reactions were reported after intra-articular injection of allogenic PB-derived MSCs in stifle joints (n=30). Whether this is attributable to the source of MSCs (PB versus bone marrow) or the type of injury remains to be determined.

The above mentioned study also reported that 24% of the horses (n=33) with stifle injuries failed to return to work within 24 months after autologous bone marrow-derived MSC treatment [6], whereas the present study reports failure to return to work in 0% (native) and 7% (chondrogenic induced) of the patients at 18 weeks after allogenic PB-derived MSC treatment (n=20). Additionally, a return to some level of work was noticed in 76% of the patients treated with native autologous MSCs, whereas in our study 80% (native) and 73% (chondrogenic) of the allogenic MSC-treated horses returned to work (score 2+3) within 18 weeks after treatment of the stifle joints. Taken both allogenic treatments together, 15 of the 20 horses (75%) returned to work, which was very similar to the 76% reported by Ferris, yet within a shorter time frame.

When comparing failure to return to work after both treatments in fetlock, coffin or pastern joints, chondrogenic induction generated more favorable outcomes, especially at short-term. Indeed, 73% of the horses with fetlock DJD were at least working after treatment with chondrogenic induced MSCs, whereas this was the case in 41% of the native MSC-treated patients. In a large prospective equine study of Lindholm, 166 arthritic fetlock joints were treated with a combination of betamethasone (12mg) and hyaluronic acid (20mg) and 68% of the joints functionally recovered within 21 days [30]. In case of equine DJD, the response to conservative treatment is being described as rather poor and short-lived [31, 32]. In this regard, it has been reported that autologous MSC-based therapies have a more prolonged therapeutic effect than corticosteroid-based treatments [33]. Indeed, most corticosteroids have a short-term functional and clinical effect until 4 weeks after injection [34, 35], whereas regenerative medicine is aiming at long-term clinical improvement. Also in the present study, both allogenic MSC-based treatments demonstrated a clear increase in average scores from 6 to 18 weeks post injection.

In the present study no statistical significant differences between both treatments could be demonstrated, since the

native MSC-treated group also generated the maximal scores in some cases and long-term average scores were very similar in both treatment groups, indicating the necessity of more power to demonstrate small differences between both treatments. This is in agreement with a previous study in rabbit knee cartilage defects where clinical, histological and immunohistochemical scores did not significantly vary between MSC- and chondrogenically predifferentiated MSC-treated joints at 3 and 6 months post injection [36]. On the other hand, it has been reported that differentiated MSC treatment of temporomandibular joint osteoarthritis improved therapeutic chondrogenic effects (histological and cartilage gene expression) in comparison with native MSCs [12]. In addition, a recent study reports inhibition of MSC chondrogenesis due to factors secreted by synovial macrophages in osteoarthritic joint synovium, and therefore, supports chondrogenic preconditioning [37]. Since it has been reported that increased numbers of MSCs are seen in the synovial fluid of patients with osteoarthritis, some sort of biological MSC response could be demonstrated [38]. In the case of aged individuals, however, a lower number and restricted chondrogenic and osteogenic differentiation capacity in progenitor cells have been reported [39, 40]. Based on all these findings, the use of an allogenic MSC therapy could offer a valuable tool for the replacement of "dysfunctional" autologous MSCs. In our study, a joint-specific beneficial effect of chondrogenic induction was noticed. In this regard, it has been reported that chondrocytes from three different equine joint types with varying prevalences in osteoarthritis differed significantly in susceptibility to apoptosis induction [41]. This may provide an indication for the joint-specific nature of the disease and the necessity for a joint-related regenerative approach. Nevertheless, future research with larger groups per joint and a longer follow-up period might provide valuable insights to this matter.

Although this study reports the functional evolution after allogenic MSC therapy in 165 equine patients with naturally occurring DJD, the exact *modus operandi* and transplantation efficacy of cell-based therapies remains to be determined. Indeed, previous studies describing autologous MSC treatment of experimentally induced cartilage lesions in equine carpal and stifle joints were not able to detect any clinical improvement [9, 42, 43]. On the other hand, an early beneficial impact on histologic appearance and biochemical composition [43] as well as a late enhancement of aggrecan levels [42] could be observed. In this regard, Frisbie *et al.* [9] reported no significant clinical or histological effect within 70 days after treatment with autologous bone marrow-derived MSCs in the middle carpal joint of horses, but observed improvements in synovial fluid PgE2 levels, which would ultimately inhibit the production of pro-inflammatory cytokines and the correlated degenerating effects of matrix metalloproteinases [44, 45]. Although this biological effect might lead to clinical improvement, additional studies should be conducted to provide more insights to this matter.

In conclusion, our results indicate overall better clinical outcomes in the chondrogenic induced MSC group, however, without statistical significance. More protracted and powered studies need to be performed to confirm superior clinical effects of chondrogenic induction on DJD in horses.

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