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CLINICAL TRIAL

Equine Allogeneic Chondrogenic Induced Mesenchymal Stem Cells Are an Effective Treatment for Degenerative Joint Disease in Horses

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Degenerative joint disease is one of the main causes of equine early retirement from pleasure riding or a performance career. The disease is initially triggered by an abnormal loading of normal cartilage or a normal loading of abnormal cartilage. This primary insult is accompanied with joint inflammation, which leads to further progressive degeneration of the articular cartilage and changes in the surrounding tissues. Therefore, in search for an effective treatment, 75 adult horses with early signs of degenerative fetlock joint disease were enrolled in a randomized, multicenter, double-blinded, and placebo-controlled study. Fifty animals were injected intra-articularly with the investigational veterinary product (IVP) consisting of allogeneic chondrogenic induced mesenchymal stem cells (ciMSCs) with equine allogeneic plasma, and 25 horses were injected with 0.9% NaCl (saline) control product. From week 3 to 18 after treatment, lameness scores ($P < 0.001$), flexion test responses ($P < 0.034$), and joint effusion scores ($P < 0.001$) were remarkably superior in IVP-treated horses. Besides nasal discharge in both treatment groups, no adverse events were observed during the entire study period. On long-term follow-up (1 year), significantly more investigational product-treated horses were working at training level or were returned to their previous level of work ($P < 0.001$).

Keywords: allogeneic, MSC, arthrosis, horse, GCP, field trial

Introduction

Just like in human medicine, degenerative joint disease is a well-known and common problem in equine medicine. The disease starts with an injury or pathology in the soft tissues, subchondral bone, or articular cartilage of the joint or with a combination of the above. This initial trigger leads to progressive degradation of the articular cartilage together with changes in the bone and the surrounding soft tissues [1]. Whatever the primary cause may be, joint inflammation or, more specifically, synovitis is an important aggravator or mediator of degenerative joint disease. Indeed, synovitis leads to the production of proinflammatory cytokines and matrix-degrading enzymes, contributing to the disease development [2,3]. Moreover, synovitis causes pain and joint effusion, which contributes to joint (micro-)instability.

Approximately 25% of horses are affected with some stage of degenerative joint disease at some point of their lifetime, be it an early stage mainly characterized by an inflammatory component and altered cartilage metabolism or a more chronic stage characterized by bony changes with intermittent inflammatory flares and severe cartilage destruction [4,5]. Considering that there are ~5.7 million horses registered in official databases in Europe [6] and 9.2 million in the United States [7], and that, according to an official European market study from Euromonitor, ~25% of injured horses visit a

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veterinarian regularly, 356,000 European horses and 575,000 American horses that visit a veterinarian suffer from osteoarthritis at a certain point in their life.

Currently, in both human and veterinary medicine, different treatments are available to ease the pain and increase the patient's comfort. Similar to human medicine, the most frequently used medicinal products in equine practice for early-stage joint inflammation are nonsteroidal anti-inflammatory drugs (NSAIDs), corticosteroids, glucosamine, chondroitin sulfate, and hyaluronic acid [8–10].

NSAIDs moderately reduce the pain and inflammation associated with degenerative joint disease. However, they require daily administration and gastrointestinal and kidney problems are well-known side effects [10,11]. Corticosteroids effectively reduce inflammation and pain, but often have to be administered on repeated occasions to sustain their effect. Moreover, corticosteroids are sometimes disputed, since they potentially have a detrimental effect on the already compromised cartilage metabolism [5,11]. In addition, they are most effective when there is a strong inflammatory component and less when the main problem is tissue degradation.

Hyaluronic acid has been reported to reduce inflammation, especially when administered intravenously. However, when administered intra-articularly, this effect is far less pronounced and it has been reported to cause flare reactions [11]. Because of these flare reactions, hyaluronic acid is often combined in practice with corticosteroid for intra-articular administration. A recent study on intra-articular use of hyaluronic acid has, however, reported no benefit of using hyaluronic acid combined with triamcinolone over triamcinolone alone [12].

Glucosamine and chondroitin sulfate are available as nutraceuticals and are popular in equine practice [13]. However, since nutraceuticals are not regulated, they are not standardly tested on safety and efficacy, and often information on their oral bioavailability is lacking [8,11].

Thus, currently, most treatments focus on reducing the symptoms of degenerative joint disease, but not to prevent further degradation. More recently, in search for treatment alternatives, cell-based therapies are being investigated because of their biological nature and regenerative potential [14–17]. Indeed, there are indications that intra-articular application of mesenchymal stem cells (MSCs) improves cartilage healing [14–16]. In addition, in vitro observations have demonstrated that chondrogenic induced MSCs produce cartilage-specific substances, such as aggrecan, glycosaminoglycans, and collagen type II, which could aid cartilage repair [17–20].

Despite the mode of action of MSCs being largely undefined, several mechanisms observed in in vitro and animal experiments are assumed to be responsible for the effect seen in patients [21]. The classic hypothesis that MSCs migrate to the site of injury, integrate in the tissue, and differentiate into functional cells is being progressively abandoned. Currently, it is inferred that MSCs heal injured tissue by using paracrine signals, cell-cell contact through nanotubes, cell fusion events, or by the secretion of extracellular vesicles [21,22]. These mechanisms are deemed responsible for the anti-inflammatory and angiogenic effect MSCs display under certain circumstances and the reason they can stimulate local cell survival and proliferation [21,22].

MSC-based therapies would thus potentially provide a more durable solution to degenerative joint disease, since they can possibly stimulate local cartilage repair and thus retard or even reverse the disease process. However, because experimental models do not completely resemble clinical pathology of naturally occurring degenerative joint disease, veterinary patient studies using autologous or allogeneic MSCs have also been conducted. These studies report the safety and efficacy of stem cell treatments based on an average amount of horses that were able to return to work or to return to previous levels of performance, which varied from 76% to 86% depending on the affected joints [23,24]. Nevertheless, these studies were not blinded and did not include control groups.

Thus, although horse owners hesitate to participate in clinical trials with placebo treatments, it is imperative to perform double-blinded superiority (compared to a placebo) or noninferiority (compared to a registered medicinal treatment) clinical trials to evaluate a new therapy for its effectiveness.

Veterinary MSC treatments are defined as a veterinary medicinal product according to the pharmaceutical act of the EU [Art. 1 No. 2 Directive 2001/82/Ethics committee (EC)] [25] and are thus subjected to strict regulations. In human medicine, 1,052 novel clinical stem cell trials have been identified so far, but only 3.5% resulted in a successful marketing authorization [26], indicating the difficulty to demonstrate evidence-based efficacy and/or safety of a cell-based therapy.

To the authors' knowledge, in veterinary medicine, no clinical field trial for cell-based products in equine orthopedics has been reported to date. However, to prove safety and efficacy of a novel stem cell-based product in horses, a field trial should be conducted compliant to Good Clinical Practice (GCP), as described by the Veterinary International Conference on Harmonization (VICH) Guideline number 9 [Committee for Medicinal Products for Veterinary Use (CVMP)/VICH/595/98]. Agreed by EU, Japan, and the United States, this quality standard provides detailed guidance on the requirements of clinical studies needed to obtain marketing authorization of new veterinary medicinal products in these markets.

Thus, based on this guideline, a field trial should be controlled, double blinded, multicenter, and randomized to effectively evaluate efficacy and safety of a cell-based therapy for treatment of naturally occurring degenerative joint disease in horses.

Therefore, to accommodate current legislation and to address the scientific need for a more durable solution, a placebo-controlled, double-blinded, multicenter, randomized GCP-compliant clinical field trial was performed, evaluating the safety and efficacy of equine MSCs as a treatment for naturally occurring degenerative joint disease (or chronic joint inflammation as an early stage of degenerative joint disease) in horses.

Since it has been reported that the microenvironment in inflamed joints has an influence on the paracrine signaling of equine MSCs [27], the MSCs used in this study were chondrogenically primed to stimulate the cells to produce the correct paracrine substances. Moreover, equine allogeneic plasma (EAP) was added to the MSCs before injection, because this has been shown to increase clinical improvement of horses with degenerative joint disease of the fetlock [17].

The hypothesis of this study was that allogeneic chondrogenic induced MSCs (ciMSCs) combined with EAP

would be a safe and effective treatment, and would have superior and clinically relevant outcome compared to a placebo (saline) for the treatment of mild-to-moderate (early) degenerative fetlock joint disease in 75 horses.

Materials and Methods

Regulatory requirements and animal welfare regulations

This study was carried out in accordance with recommendations of the Animal Welfare Department of the Belgian Federal Public Service of Health. The study protocol was approved (EC_2015_003) by the Ethics Committee of Global Stem cell Technology (Permit Number: LA1700607). In addition, the study was conducted according to European and national regulatory requirements and in compliance with Directive 2001/82/EC, VICH GL9 (GCP, June 2000), EMA/CVMP/Efficacy Working Party (EWP)/81976/2010 (Guideline on statistical principles for clinical trials for veterinary medicinal products).

The medicinal products in this trials were produced according to Good Manufacturing Practice (GMP) (certificates: BE/GMP/2015/082 and BE/GMP/2016/069) and with manufacturing authorization 1868V for veterinary medicinal products. The field study was approved and received clinical trial authorization permit 0002829 from the Belgian federal agency for medicines and health products. Before enrolment, written owner consent was obtained from each owner of horses participating in the study.

Investigational veterinary product and control product

The investigational veterinary product (IVP) consisted out of a proprietary combination of allogeneic ciMSCs and EAP. Preparation of the IVP is briefly described below. Saline (0.9% NaCl = Vetivex 9 mg/mL; Dechra Limited, Staffordshire, United Kingdom) was used as the control product (CP).

Isolation and chondrogenic induction of MSCs. In total, 50 mL of blood was collected in sterile ethylenediaminetetraacetic acid (EDTA) tubes from the *vena jugularis* of a 6-year-old donor gelding, which was tested for 32 different transmittable diseases at Böse laboratory (Harsum, Germany), in agreement with the European Medicines Agency (EMA) requirements. This donor horse was further not involved in the study in any way. Approval of the ethics committee was obtained for blood sampling of the donor horse (EC_2012_001 and EC_2016_003).

Isolation, characterization, and freezing of the intermediate cell stock were performed at P5 as described previously [20]. Cells were thawed, cultured, and subsequently chondrogenically induced from P9 to P10 using a proprietary method and media. The cells were characterized by assessing the total cell number, viability, and sterility, gene expression of a chondrogenic marker (cartilage oligomeric protein: COMP), and the presence of cell surface markers [cluster of differentiation (CD45), major histocompatibility complex (MHC) II, CD29, CD44, and CD90].

Chondrogenic induced MSCs were trypsinized, resuspended in 1 mL of Dulbecco's modified Eagle's medium low glucose (DMEM LG) with 10% of dimethyl sulfoxide (Sigma) at a concentration of 2×10^6 cells per mL, and frozen before being shipped on dry ice for clinical application. Viability and gene expression of COMP were again assessed after 6 and 12 months of frozen storage to assess ciMSC batch stability.

Preparation of EAP. In total, 900 mL of peripheral blood was collected from a single donor horse (a gelding of 14 years) in a citrate phosphate dextrose adenine-1 single blood bag (Terumo®) for EAP preparation. This donor horse was a different individual from the stem cell donor, but was also tested for 32 different transmittable diseases at Böse laboratory, in agreement with the EMA requirements. This donor horse was also further not involved in the study in any way.

One hundred samples of 1 mL EAP were prepared as previously described by our group [28,29]. Each sample contained $\sim 85 \times 10^6$ platelets and was frozen and stored at −80°C until clinical application.

Patient inclusion and exclusion criteria

In total, 75 warmblood horses, 3 to 23 years of age, with recurrent lameness were enrolled in this study: 22 mares, 16 geldings, and 37 stallions. The MHC status of each individual patient was not determined. However, horses were not expected to be MHC matched with our MSC donor. Thus, most likely, horses were semiallogeneic or full allogeneic for MHC molecules.

To be included, horses had to present grade 2 or 3 lameness on the American Association of Equine Practitioners (AAEP) scale associated with (early staged) fetlock degenerative joint disease lasting for at least 2 months (early staged degenerative joint disease was defined as joint inflammation lasting over 2 months). In addition, lameness had to be confirmed by a positive intra-articular anesthesia of the fetlock and a positive flexion test. The fetlock joint also needed to show at least one sign of inflammation (swelling, pain on palpation, or heat assessed by palpation).

Horses were excluded if they received an unauthorized pretreatment (eg, corticosteroids), which still could have an effect on the pathology, if they had a severe medical condition that, in the opinion of the investigator, would have compromised their safe participation in the study, and if the horses had any condition, actual or anticipated, which the investigator felt would restrict or limit their successful participation for the entire duration of the study.

Other exclusion criteria consisted of previous participation in a stem cell study with the treated joint, lameness on more than one limb, AAEP scores of 1, 4, or 5, or lameness due to any other locomotion problem (nervous system and back problem). Moreover, a narrowed joint interspace reducing ≥1/3 of the normal fetlock joint space on lateromedial (LM) or dorsoplantar (DP) X-ray was not allowed. All horses were withdrawn from medication from study start to study completion. In addition, no therapies were offered at the study end.

Blinding and randomization

Due to the color difference between the IVP and the CP, the study was blinded by using separate personnel for clinical examinations (investigator and examining veterinarian) and administration of treatments (dispenser); so there were two

separate teams (examining vet and dispenser) at both study sites (two veterinary clinics) performing the different tasks within the study. Owners were not present when the treatment was administrated, so they were also blinded to which treatment their horse had received. A random treatment allocation plan was prepared for each study site and provided separately to the dispenser. The investigator/examining veterinarian assigned a unique ID number to the animal. The random treatment allocation plan was created using a block size of 3, whereby a ratio of 2:1 horses (IVP:CP) was allocated to the treatment groups.

Treatment and rehabilitation protocol

On day 0, all inclusion and exclusion criteria were evaluated, an intra-articular anesthesia was performed on the affected joint, and LM and DP X-rays were taken. The horses were treated ~24 h later (day 1) to avoid mixing between anesthetics and equine MSCs in the joint, which could cause severe cell damage [30]. On day 1, each horse was sedated using detomidine hydrochloride (Detonervin 10 mg/mL, 0.5 mL IV; Le Vet B.V., The Netherlands), and ketoprofen (Ketofen 10%; Merial Animal Health, Belgium, 10 mL IV) was given as concomitant nonsteroidal anti-inflammatory treatment before intra-articular application of 2 mL of the IVP or 2 mL of the CP. For the IVP administration, both the EAP (1 mL) and ciMSCs (1 mL) were thawed at 25°C–37°C and drawn into one syringe. The IVP was injected immediately after mixing.

The first 3 days of the study, the horses were walked up and down a corridor only. After that, they were walked with the rider up to 1 week, followed by walking and trotting with the rider up to the first clinical evaluation at week 3. Depending on the clinical status of the horse, they gradually returned to work by including canter exercises on week 4 and return to full work at week 6 or continued restricted walking and trotting exercises up to week 6. The rehabilitation occurred in the owners care or at the veterinary clinic the first 3 days of the study. The remainder of the study, the rehabilitation occurred in the owners care.

Table 1. Overview of the Score Systems Used by the Examining Veterinarians for Assessing Lameness, Response to Flexion Test, Joint Effusion, Heat at Palpation, and Pain to Pressure, and the Scores Attributed by the Owners During the Owner Questionnaires

Parameter	*Score*	*Clinical implication*
	Veterinary scoring	
AAEP grading	0	No lameness
	1	Lameness not consistent, regardless of circumstances
	2	Lameness consistent under certain circumstances
	3	Lameness consistently observable on a straight line
	4	Obvious lameness: marked nodding or shortened stride
	5	Minimal weight-bearing lameness in motion or at rest
Flexion test	0	No flexion response
	1	Mild flexion response
	2	Moderate flexion response
	3	Severe flexion response
Joint effusion	0	No swelling
	1	Mild swelling
	2	Moderate swelling
	3	Severe swelling
	4	Extreme swelling (also periarticular)
Heat at palpation	0	No increased temperature sensation
	1	Mild increased temperature sensation
	2	Moderate increased temperature sensation
	3	Severe increased temperature sensation
Pain to pressure	0	No pain to pressure
	1	Mild pain to pressure
	2	Moderate pain to pressure
	3	Severe pain to pressure
	Owner scoring	
Horse improvement	0%	Not at all
	20%	Marginal improvement
	40%	Mild improvement
	60%	Moderate improvement
	80%	Remarkable improvement
	100%	No more discomfort noticeable
Current working status	00	Failure to return to work
	0	Rehabilitating
	1	Return to work
	2	Return to previous level of work

AAEP, American Association of Equine Practitioners.

Evaluation protocol

On day 0 and at week 3 and 6, a visual lameness assessment and flexion test were performed on all horses included in the study, using the AAEP score system and the score system depicted in Table 1, respectively. On day 1 and 2, a reduced lameness examination was performed by walking the horse up and down a corridor and evaluating lameness based on the following scores:

- no lameness visible (score 0),
- lameness difficult to observe and not consistent (score 1)
- lameness obvious at walk (score 2)
- minimal weight bearing (score 3).

Based on other clinical studies [17,23], week 6 was defined as the time point to evaluate the primary efficacy endpoint to observe a sustained clinical effect of treatment. A relevant clinical improvement, the primary efficacy criterion, was considered a reduction of AAEP lameness score from 2 or 3 (clear clinical lameness) at inclusion to an AAEP score of 0 or 1 (no or inconsistent clinical lameness). The total duration of the study per animal was allowed to range from 37 to 47 days (day 0 to week 6 ± 5 days). At week 6, the owners were also consulted to rate the condition and improvement of their horse (Table 1). Further veterinary evaluation (joint and lameness assessment scored according to Table 1) was planned for week 12 ± 1 week and week 18 ± 1 week. In case the patient could not be presented to the investigator, an owner questionnaire was used to obtain data from the patient. In addition, at week 18 and 1 year after treatment, owners were contacted again to inform about the work status of their horse.

Any observation in animals that was unfavorable and unintended, and occurred after the use of the IVP or CP was defined an adverse event (AE). A suspected adverse drug reaction was defined as an AE where a relation to treatment was suspected. A serious adverse event (SAE) was defined as any AE that resulted in death, was life-threatening, or resulted in persistent or significant disability/incapacity. As lameness and joint abnormalities in the treated affected limb were recorded separately as they were part of the efficacy evaluation criteria, these were not documented separately as AEs.

A clinical examination was performed of each horse on day 0, 1, and 2 and at week 3 and 6, and whenever requested by the owner, and consisted of temperature, respiratory rate, and heart rate measurements combined with a general body examination. Local clinical inflammatory parameters, such as heat at palpation, pain to pressure, and joint swelling were also scored at these time points (Table 1).

Statistical analysis

The sample size of the study was calculated using SAS® statistical analysis software (Version 9.3) of the SAS Institute, Inc. (Cary, NC), so a two-group χ^2 test with a 0.05 two-sided significance would have 80% power to detect the difference between a control proportion, π_1, of 0.3 (30% estimated success) and an IVP proportion, π_2, of 0.6 or 0.7 (60%–70% estimated success). Based on this calculation with an unbalanced distribution of group (IVP:CP = 2:1), at least 50 animals in the IVP treatment group and 25 animals in the CP per-treatment group were shown to be sufficient to demonstrate potential statistical superiority.

All data were collected on pre-established data capture forms. Then data were entered to a specifically established electronic database, verified, and inconsistencies sorted. Statistical analysis was performed on the data transferred from that database to SAS statistical analysis software (Version 9.3) of the SAS Institute, Inc. The difference in relevant clinical improvement scores was compared between groups at different time points using Fisher's exact test. To compare all clinical and owner scores presenting number and percent of each score category, the Mantel–Haenszel test was used. The difference in working status was compared using Mann–Whitney *U* test. The percentage of animals with AEs was compared between groups using Fisher's exact test. A 5% level of significance was used to assess statistical differences.

Results

Isolation and characterization of MSCs

The intermediate cell stock displayed all properties to be characterized as MSCs. Briefly, they attached to plastic, trilineage differentiation was performed successfully, and MSCs were positive for CD29 (100%), CD44 (100%), and CD90 (100%) and negative for CD45 (1%) and MHC II (0%) (Fig. 1). The average population doubling time over 10 passages was 1.4 and passage 10 ciMSCs displayed 96% viability, an MSC immunophenotype (100% CD29, 87% CD44, 98% CD90, 2% CD45, and 0% MHC II), and a 4.4-fold COMP increase as a marker for chondrogenic induction [17]. After 6 months of frozen storage, 82% viability and 4.6-fold COMP change were present. At 12 months, viability remained above 80% and a 4.2-fold COMP change was present. All horses were treated within this period after MSC production.

Clinical outcome

All 75 horses included in this study showed initially a moderate lameness (score 2–3 out of 5 on the AAEP scale; Fig. 2), mild-to-moderate response to flexion test (score 1–2 out of 3; Fig. 3), and mild-to-moderate joint swelling (score 1–2 out of 4; Fig. 4). The clinical signs in both treatment groups upon inclusion were comparable and did not show any significant difference between groups (Table 2).

At week 3 after treatment, the AAEP lameness score was significantly ($P < 0.001$) improved in the IVP group compared to the CP group with relevant clinical improvement (decrease to AAEP score 0 or 1) in 70% of the animals (Fig. 2). At week 6, relevant clinical improvement as a primary efficacy criterion was observed in 78% of the IVP-treated horses in comparison to 24% in the CP-treated horses (Fig. 2). This difference of 54.0% was significant, and superiority was shown for IVP compared to CP ($P < 0.001$). The evaluation of the AAEP lameness scores at week 12 (92% vs. 36%) and 18 (84% vs. 17%) statistically ($P < 0.001$) confirmed a long-term clinical improvement in the IVP group when compared to the CP group (Fig. 2). In the CP-treated group, 64% of the animals still showed a lameness score of 2 or 3 at week 12 and 83% at week 18 (Fig. 2). The onset of a clinically relevant improvement with the IVP treatment was shown as early as week 3, and this effect continued until week 18.

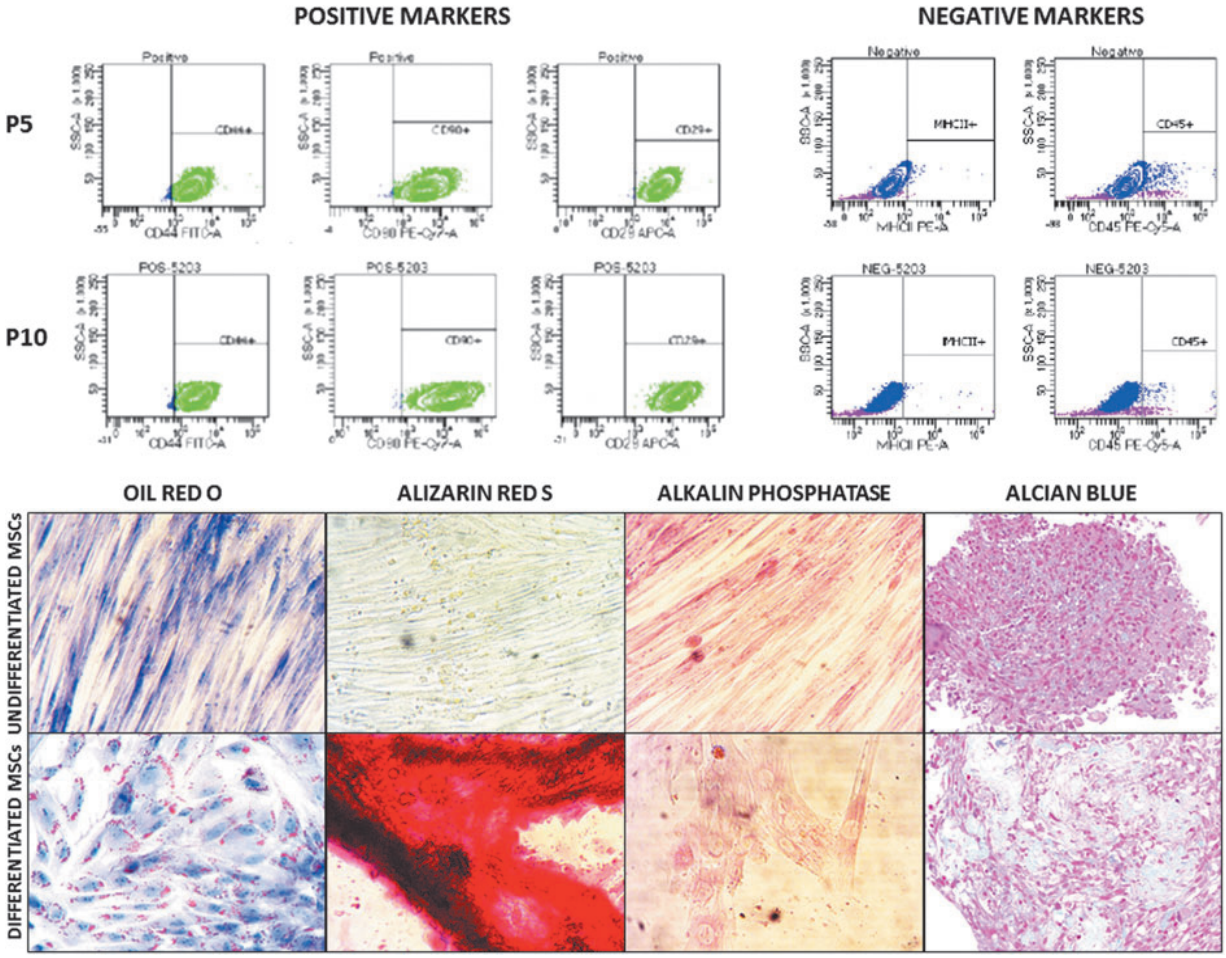

FIG. 1. Representative flow cytometric images of positive (CD29, CD44, and CD90) and negative (CD45 and MHC II) MSC markers. Adipogenic differentiation was confirmed with Oil Red O staining, osteogenic differentiation with alizarin red S and alkaline phosphatase staining, and chondrogenic differentiation with alcian blue staining. Differentiated cells demonstrated morphological changes and positive staining areas, whereas undifferentiated cells remained spindle shaped without convincing staining. CD, cluster of differentiation; MHC, major histocompatibility complex; MSC, mesenchymal stem cell.

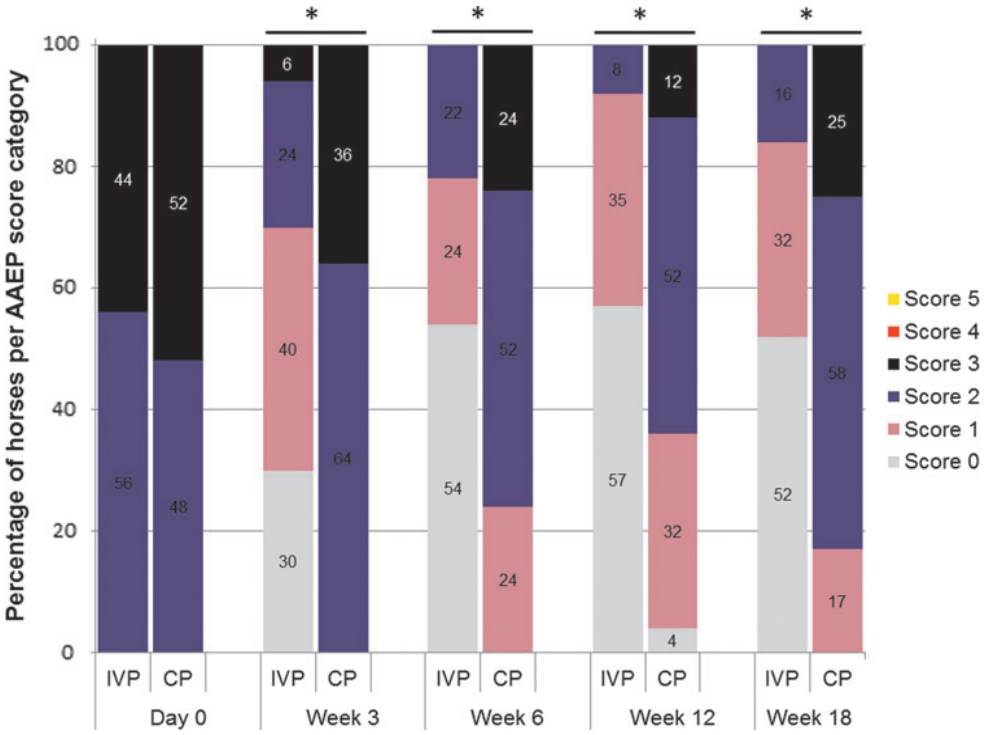

FIG. 2. The distribution of horses (in percentages) over the AAEP score categories per time point for the IVP-treated group and placebo control (CP)-treated group. The asterisk (*) indicates a significant difference in frequency of scores between the two treatments groups ($P < 0.001$). AAEP, American Association of Equine Practitioners; CP, control product; IVP, investigational veterinary product.

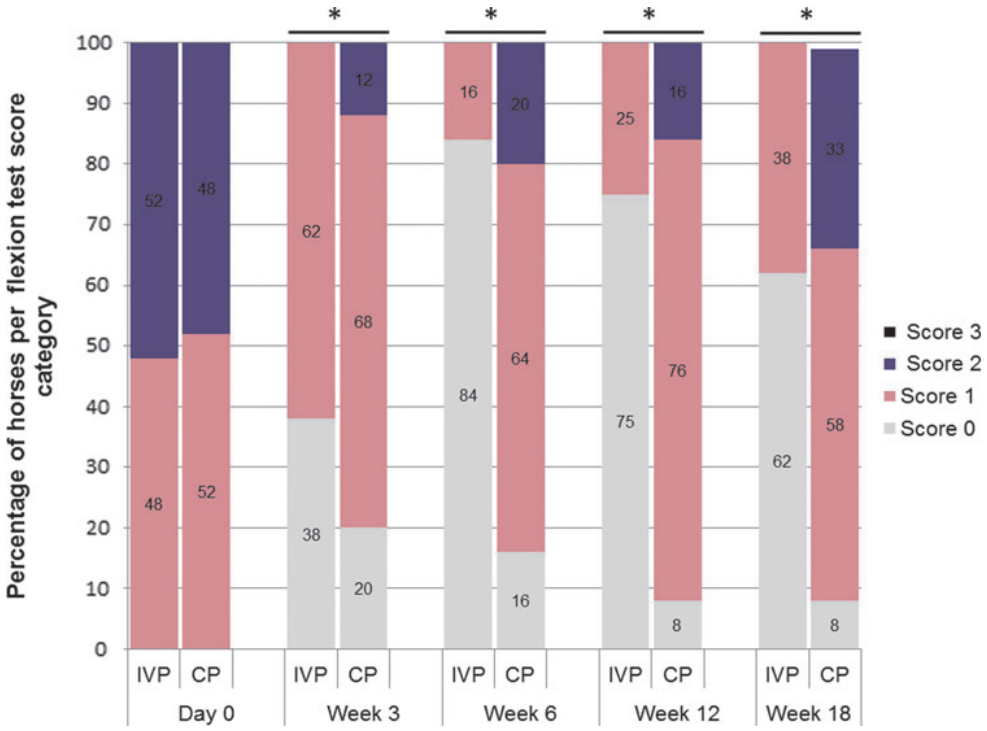

FIG. 3. The evolution of the percentage of horses per flexion test score category over time for the IVP-treated group and placebo control (CP)-treated group. The asterisk (*) indicates a significant difference in frequency of scores between the two treatments groups ($P < 0.05$ for week 3 and $P < 0.001$ for weeks 6, 12, and 18, respectively).

The distribution of scores of the secondary efficacy criterion flexion test response and joint swelling was significantly different between groups at all postbaseline examinations, week 3, 6, 12, and 18, with lower scores in the IVP-treated animals than in CP-treated animals (Figs. 3 and 4). At week 3, no flexion response was observed in 38% of IVP-treated horses, which was significantly higher than the 20% of CP-treated horses ($P < 0.05$) (Fig. 3). In the CP group, the percentage of animals with a negative response to flexion decreased over time toward 8% of the horses at week 12 and 18 (Fig. 3). In the IVP-treated group, the percentage of negative flexion response was 84% at week 6 and reduced to 75% at week 12 and 62% at week 18, which was significantly better than in the animals of the CP group at all time points ($P < 0.001$) (Fig. 3).

At week 6, owners rated at least an 80% improvement in 72% of the IVP-treated animals. This was significantly more ($P < 0.001$) than for the CP-treated horses (Table 3 and Fig. 5). At week 12 and 18, one horse of the IVP and CP group, respectively, was not presented for follow-up examination. The owner questionnaire indicated that one of these animals

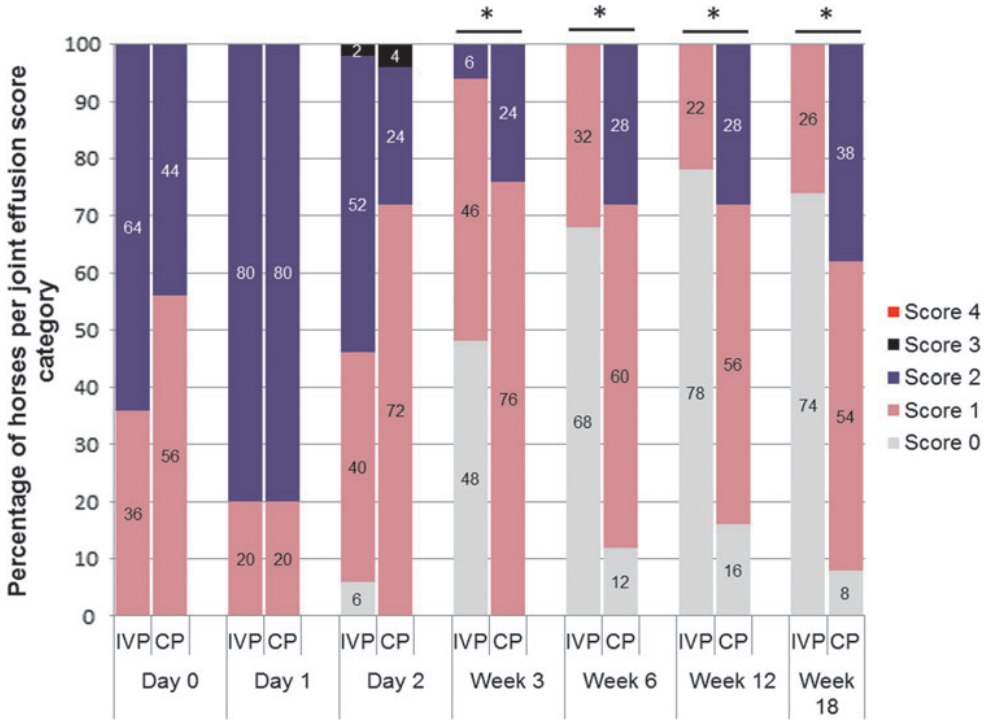

FIG. 4. The distribution of horses (in percentages) over the joint effusion score categories per time point for the IVP-treated group and placebo control (CP)-treated group. The asterisk (*) indicates a significant difference in frequency of scores between the two treatments groups ($P < 0.001$).

Table 2. Evaluation of Inclusion Criteria for Both Treatment Groups

Baseline parameters	*IVP (N = 50)*	*CP (N = 25)*	*Comparison P value*
Age in years			
Mean (SD)	9.9 (3.42)	10.1 (4.81)	0.702[a]
Min–max	3–17	5–23	
Median	10.0	9.0	
Q1–Q3	7.0–12.0	6.0–12.0	
Gender, *n* (%)			
Gelding	11 (22)	5 (20)	0.152[b]
Mare	18 (36)	4 (16)	
Stallion	21 (42)	16 (64)	
AAEP score, *n* (%)			
2 = lameness difficult to observe	28 (56)	12 (48)	0.516[c]
3 = lameness consistently observable	22 (44)	13 (52)	
Flexion score, *n* (%)			
1 = mild response	24 (48)	13 (52)	0.746[c]
2 = moderate response	26 (52)	12 (48)	
Pain score, *n* (%)			
0 = no pain to pressure	37 (74)	16 (64)	0.373[c]
1 = mild pain to pressure	13 (26)	9 (36)	
Heat score, *n* (%)			
0 = no increased temperature	31 (62)	16 (64)	0.867[c]
1 = mild increased temperature	19 (38)	9 (36)	
Swelling score, *n* (%)			
1 = mild swelling	18 (36)	14 (56)	0.101[c]
2 = moderate swelling	32 (64)	11 (44)	

[a]*P* values are based on the Wilcoxon rank-sum statistic.
[b]*P* value is based on the Fisher's exact test.
[c]*P* values are based on the Mantel–Haenszel chi-square statistic.
AAEP, American Association of Equine Practitioners; CP, control product; IVP, investigational veterinary product; Q, Quartile; SD, standard deviation.

returned to work (IVP treatment) and the other animal was still rehabilitating (CP group).

There was a significant difference in working status of animals between the IVP and CP group ($P < 0.001$) at every time point after baseline (Fig. 6). At week 6, 30% of the horses in the IVP group already returned to their previous level of work compared to none of the horses in the CP group. In addition, 42% of the horses in the IVP group were working at training level compared to none of the CP group horses. At week 18, the percentage of horses that returned to their previous level of work increased to 42% in the IVP group, while in the CP group, this was still 0% (Fig. 6). Therefore, at week 18, in the IVP group, 82% of the horses returned to some level of work (working at training level+returned to previous level) compared to 16% in the CP group. One year after treatment, in the IVP group, 37% of the horses were working at training level compared to 8% of the horses treated with CP. Moreover, 47% of the horses treated with IVP returned to their previous level of work, compared to none of the horses treated with CP (Fig. 6).

Table 3. Distribution of Horse Improvement Scores as Indicated by the Owners at Week 6 After Treatment

Score	*IVP (N = 50)*	*CP (N = 25)*	*Total (N = 75)*	*Comparison P value*
Horse improvement				
0%	1 (2%)	9 (36%)	10 (13%)	<0.001
20%	6 (12%)	10 (40%)	16 (21%)	
40%	4 (8%)	0 (0%)	4 (5%)	
60%	3 (6%)	6 (24%)	9 (12%)	
80%	16 (32%)	0 (0%)	16 (21%)	
100%	20 (40%)	0 (0%)	20 (27%)	

Concomitant medication after study completion (week 6), that is, a single intravenous NSAID injection, was necessary for two animals (4%) in the IVP group and for nine animals (36%) in the CP group, which was statistically significant ($P < 0.001$), yet did not substantially influence the later scorings of these animals at week 12 and 18.

In total, three AEs were observed in 3 animals out of 75 allocated to treatment, all diagnosed as mild infections of the upper respiratory tract showing nasal discharge. Two of the AEs were observed in the IVP group and one in the CP group (4.0% in both groups) with no statistically significant difference between groups ($P = 1.000$). Neither of the AEs was serious nor regarded as related to the study treatment.

At the reduced lameness assessment on day 2, no statistical significant difference between groups was observed ($P = 1.000$).

There was no significant difference between groups in the frequency of local reactions (joint effusion, heat at palpation, and pain to pressure) within the first 2 days after treatment ($P > 0.999$). In one animal (2%) in the IVP group, joint swelling and heat at the injection site worsened from day 1 to 2 by one score point and pain to pressure worsened in another animal (2%) from day 1 to 2 by one score point. In the CP group, increased joint swelling was observed in

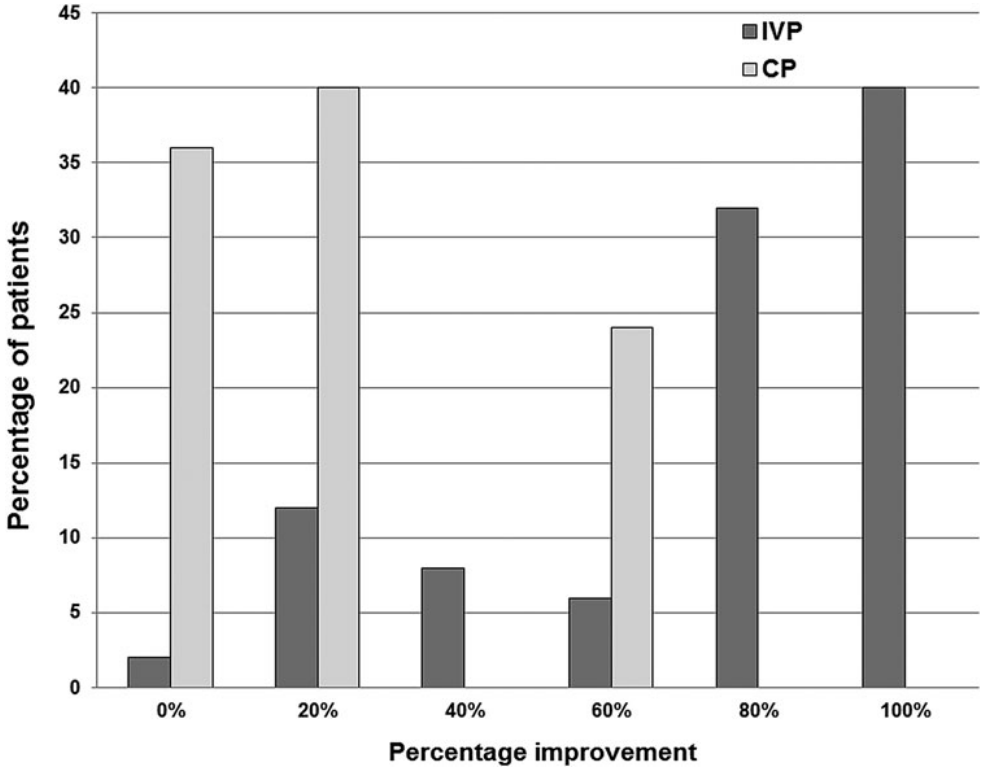

FIG. 5. The distribution of the horses (in percentages) over the clinical improvement categories indicated by the owners at week 6 for both treatment groups. There was a significant difference ($P < 0.001$) between the IVP and placebo control (CP) group for each score category.

one animal (4.0%) from day 1 to 2. From week 3 to 18 onward, animals in the IVP group demonstrated significantly ($P < 0.001$) less joint effusion, of which 68%–78% of the horses were without joint swelling from week 6 onward in comparison to 49%–57% in the CP group (Fig. 4).

The presence of pain to pressure at the injection site was not significantly different at observations at week 3 and 6 ($P \geq 0.071$) with the majority of animals in both groups (ranging from 88.0% to 100%) showing no local pain to pressure. Although significantly lower scores were observed in the IVP group than in the CP group at weeks 12 and 18 ($P < 0.015$), the majority of animals (ranging from 87.5% to 100%) of both groups showed no local pain to pressure at these observations. Except for week 6 ($P < 0.05$), the presence of heat at the injection site was not significantly different between groups on any postbaseline day ($P > 0.149$).

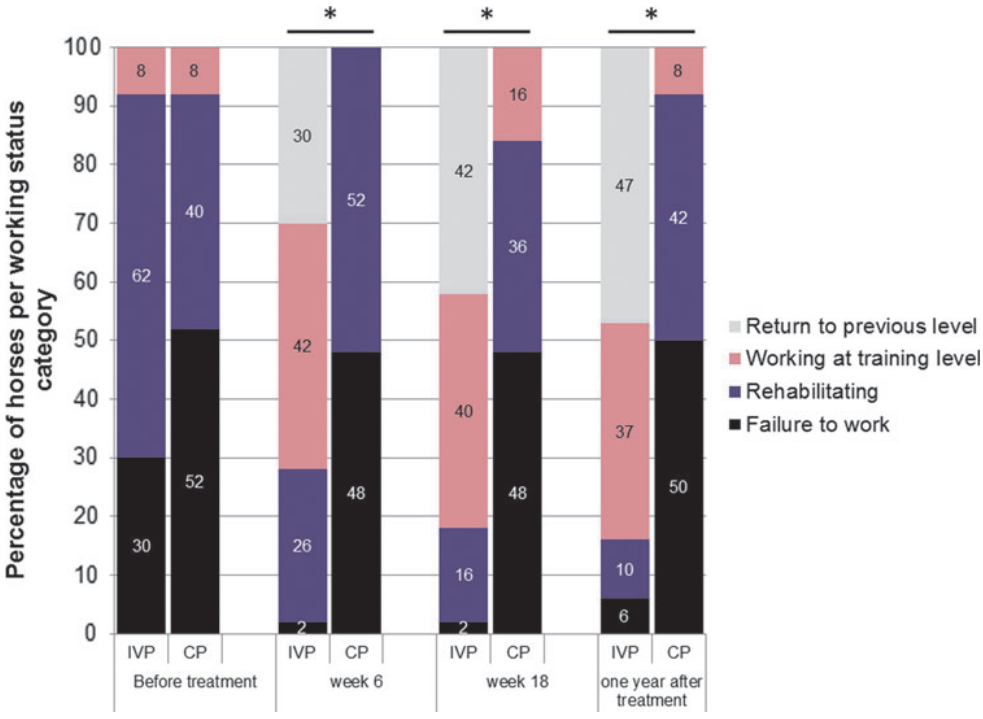

FIG. 6. The distribution of the horses (in percentages) over the working status categories indicated by the owner per time point for the IVP-treated group and placebo control (CP)-treated group. The asterisk (*) indicates a significant difference in frequency of scores between the two treatments groups ($P < 0.001$).

However, the majority of animals in both groups (ranging from 95.8% to 100%) showed normal temperature at the injection site.

Discussion

In this study, 75 horses with naturally occurring fetlock joint inflammation were enrolled, of which 50 horses were treated with allogeneic chondrogenic induced MSCs combined with EAP (IVP) and 25 with a placebo, being 0.9% NaCl (CP). Such negative controlled study tends to be a challenge, especially to convince owners to participate, but was necessary due to the lack of a licensed treatment suitable for comparison in this study. In this study, exit criteria had been defined to allow withdrawal of animals from the study in case of relevant pain, so owners were reassured their horse would not have to suffer from unnecessary pain if enrolled in the study. An ethical committee had reviewed the study protocol before implementation.

Relevant clinical improvement and effusion scores increased or remained stable in the IVP-treated group from week 3 toward week 18, whereas the opposite trend was present in the CP-treated horses. Interestingly, 20%–25% of the saline-treated horses also demonstrated relevant clinical improvement at a certain time point. This could be due to the adjusted training program or to the therapeutic effect of saline as CP, which has been reported previously [31]. Nevertheless, for all observed parameters, clinical improvement decreased over time in the CP group. In the IVP group, the negative joint flexion also decreased from 84% of the horses at week 6 to 62% of the horses at week 18. However, at the long-term follow-up of 1 year, 84% of the horses in the IVP group were working at training level or their previous level, indicating a long-term sustainability of the IVP-treated joints.

In this study, EAP from a single donor horse was added to the ciMSCs, which is meant to improve MSC proliferation and chondrogenic differentiation [32]. Indeed, when using EAP, the manufacturing process needs to be well controlled and standardized to produce a reproducible product and to reduce white and red blood cells as much as possible to prevent transfusion-related erythrolysis. In this study, a single injection of allogeneic EAP and MSCs was performed and did not cause any sign of product-related AEs. Nasal discharge was observed in some animals of both groups, indicating typical seasonal disease during the fall and winter months. They were considered not to be related to the treatment.

In general, allogeneic MSCs are being considered equally immunomodulating as autologous MSCs in vitro [33,34] and in vivo [35,36], but it has been reported that repeated intra-articular administration of allogeneic equine MSCs may cause a significant increase in total nucleated cell count [37]. This increase was still within acceptable clinical ranges [38] and another study reported no AEs after repeated injection of pooled allogeneic MSCs [39]. However, it should be considered that MSCs are heterogeneous in MHC II expression [40], and should therefore be tested in vitro before clinical application, as reported in this study. Moreover, certain inflammatory parameters can upregulate MHC molecules, which increases the chances for graft rejection [40,41]. This could also explain the increase in total nucleated cell count as mentioned above [37].

In this regard, a recent study reported a reduced MHC expression after pretreating MSCs with transforming growth factor-β (TGF-β) [42]. Since this happens to be one of the cartilage stimulating growth factors used in this study to chondrogenically predifferentiate the MSCs, it might explain why a very low number of horses presented with clinical signs of inflammation after the injection. Moreover, the MHC I level on peripheral blood-derived MSCs is below 10% [17], offering another explanation for the low number of horses with clinical signs of inflammation in this study.

The patients in this study were also not matched with the donor MSCs and since no clinical problems were detected, this further confirms the low immunogenicity of the donor cells. Nevertheless, it should be mentioned that all horses in this study received a single injection of ketoprofen (NSAID) on the day of intra-articular injection, which may have masked a potential increase of nucleated cells; horses were injected only once with the IVP. Further research with repeated injections using the IVP without NSAID administration should provide more insights in this matter and determine whether the same safety and efficacy can be observed after repeated injections. It would also be interesting to evaluate antibody response in future studies and determine whether the same clinical results would be achieved with another donor horse (with planned donor/acceptor mismatch) or another dose.

Another important and underestimated aspect of the use of regenerative medicinal products is the use of an optimal dose. Consideration of the lowest effective dose for allogeneic MSC therapy is of highest importance, because high doses (30–50 million) of allogeneic equine MSCs could induce antibody responses in vivo [43]. Based on previous clinical studies to evaluate the effect of allogeneic ciMSCs [17,23], the dose of ciMSCs used in the IVP in this study was set at 2 million cells, which is 5–25-fold lower than the reported 10–50 million MSCs used in other equine studies [15,24,37,43,44]. Similarly, it has been described in dogs that higher treatment doses (66 million) of allogeneic MSCs result in lameness and pain, whereas this was not the case with lower doses (5 million) [45].

Even though dose-dependent effects have been reported for allogeneic MSCs for the treatment of myocardial infarction in rats [46] and graft versus host disease in mice [47], others have also demonstrated a superior outcome using a lower dose of allogeneic MSCs for treatment of injured medial collateral ligaments of rat knees [48] or for the treatment of human knee osteoarthritis [49]. In addition, after an intravenous injection of allogeneic adipose-derived MSCs in cats with chronic kidney disease, dose-related adverse effects were observed [50]. After reducing the dose from 4 to 2 million MSCs per mL, limited adverse effects and clinically interesting results [51] were noticed by the authors. All these findings confirm that the selection of the dose of allogeneic MSCs needs careful consideration.

In this study, no treatment group was included that only received the excipient EAP. However, no animals were treated with EAP alone to reduce the number and use of animals. Moreover, The IVP consists of a proprietary combination of allogeneic ciMSCs and EAP. Thus, horses would never be treated with EAP alone. In addition, the main contribution of EAP to the ciMSCs is a significant increase in cell viability after thawing of frozen ciMSCs

(3%–8% increase in viability depending on storage duration of the ciMSCs $P < 0.05$: Data not shown). Because the thrombocyte specifications of EAP are within physiological range (75,000–300,000/μL) [52], no influence of the EAP on the healing process is expected.

On the other hand, when producing actual platelet-rich-plasma (PRP) with platelet levels more than double of the physiological platelet levels, a short-term significant increase in white blood cell counts, prostaglandin E2, and total protein concentrations was detectable in synovial fluid analysis within 6–48 h after injection into equine fetlock joints [53]. In the proof-of-concept study performed with the IVP (including EAP), no such findings were visible at any of the reported time points (unpub. obsvns.). Moreover, in a previous study, we demonstrated that ciMSCs in combination with allogeneic plasma result in significant increased clinical outcomes in comparison to allogeneic plasma treatment alone in fetlock joints [17].

Lameness was evaluated using visual assessment and the AAEP scoring system and not by an objective measuring tool such as a lameness locator. However, the use of this AAEP score system was just, since Keegan et al. [54] stated, ''Such objective measures may augment, but not replace results obtained by subjective evaluation of lameness in horses.'' Although the lameness locator system demonstrated to have a better interobserver repeatability than a subjective lameness examination [55,56], the system is still flawed and not accepted as the gold standard for lameness evaluation in the literature [54,55]. In addition, the lameness locator system only takes into account vertical head and pelvis movement and leaves out several different other kinematic parameters, which can indicate lameness (eg, decreased maximum fetlock extension and decreased limb retraction or protraction).

In the subjective lameness examination, however, the examining veterinarian can take all these parameters into account. Moreover, studies on the repeatability of subjective lameness examination are often based on assessment of video tapes without sound and a shot from only one angle and assessing only one circumstance, while it has been demonstrated that a full live lameness examination improves interobserver agreement of a subjective lameness examination [55,57–59].

However, recognizing the limitations of a subjective lameness examination, some preventive measures were taken to increase objectivity: (1) horses were only included if they presented with an initial lameness score of 2 or 3 on the AAEP scale (consistent lameness), (2) lameness was confirmed for that limb by intra-articular anesthesia and a positive flexion test, (3) the examiner was blinded for treatment, but not for the initial side of lameness, facilitating lameness detection for that particular limb, (4) subjective examination was performed in exactly the same way for placebo-treated and IVP-treated horses, so any bias generated during the subjective lameness examination would have been the same for the two treatment groups, and (5) all lameness examination was performed by experienced veterinarians.

In conclusion, our results indicate that 2 million allogeneic chondrogenic induced MSCs with EAP administered once in the joint has a similar safety profile and superior efficacy compared to a placebo in the treatment of inflammatory fetlock joint disease in horses. Indeed, besides nasal discharge in both treatment groups, no AEs were observed during the entire study period. Moreover, the effect of the IVP was proven for the duration of 18 weeks based on relevant clinical improvement, namely a decrease in lameness scores, a decrease in response to flexion, and a decrease in joint effusion. Furthermore, the effect of the treatment was confirmed to sustain 1 year after administration, with significantly more horses working at their previous level or at training level at this point compared to the placebo control group.

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Author Disclosure Statement

The author J.H.S. declares competing financial interests as shareholder in Global Stem cell Technology (GST) NV. S.Y.B., J.H.S., and L.V.H. are all employed by GST. S.Y.B. and J.H.S. are inventors of several pending patents owned by GST (BE2012/0656; WO2014053418A9; WO2014053420A1; PCT/EP2013/075782). G.B. and K.H. are employees of KLIFOVET AG, who was compensated by GST for regulatory consultancy and monitoring of the study. The statistical analysis was performed by an independent statistician employed by KLIFOVET AG. All other authors declare no conflict of interests. The content of this article contains the product Arti-Cell® Forte owned by GST.

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The use of equine chondrogenic-induced mesenchymal stem cells as a treatment for osteoarthritis: A randomised, double-blinded, placebo-controlled proof-of-concept study

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Summary

Background: There is a need to improve therapies for osteoarthritis in horses.
Objectives: To assess the efficacy of equine allogeneic chondrogenic-induced mesenchymal stem cells combined with equine allogeneic plasma as a novel therapy for osteoarthritis in horses.
Study design: Randomised, double-blinded, placebo-controlled experiment.
Methods: In 12 healthy horses, osteoarthritis was induced in the metacarpophalangeal joint using an osteochondral fragment-groove model. Five weeks after surgery, horses were randomly assigned to either an intra-articular injection with chondrogenic-induced mesenchymal stem cells + equine allogeneic plasma (= intervention) or with 0.9% saline solution (= control). From surgery until the study end, horses underwent a weekly joint and lameness assessment. Synovial fluid was collected for cytology and biomarker analysis before surgery and at Weeks 5, 5 + 1d, 7, 9 and 11. At Week 11, horses were subjected to euthanasia, and the metacarpophalangeal joints were evaluated macroscopically and histologically.
Results: No serious adverse events or suspected adverse drug reactions occurred during the study. A significant improvement in visual and objective lameness was seen with the intervention compared with the control. Synovial fluid displayed a significantly higher viscosity and a significantly lower glycosaminoglycan concentration in the intervention group. Other biomarkers or cytology parameters were not significantly different between the treatment groups. Significantly less wear lines and synovial hyperaemia were present in the intervention group. The amount of cartilage oligomeric matrix protein, collagen type II and glycosaminoglycans were significantly higher in the articular cartilage of the intervention group.
Main limitations: This study assessed the short-term effect of the intervention on a limited number of horses, using an osteoarthritis model. This study also included multiple statistical tests, increasing the risk of type 1 error.
Conclusions: Equine allogeneic chondrogenic-induced mesenchymal stem cells combined with equine allogeneic plasma may be a promising treatment for osteoarthritis in horses.

The Summary is available in Spanish – see Supporting Information

Keywords: horse; allogeneic; metacarpophalangeal joint; model; peripheral blood

Introduction

Osteoarthritis (OA) in horses often results in an early retirement from an athletic career or pleasure riding [1–3]. Currently, treatment of OA is mainly focused on addressing the clinical signs [1,3]. The most commonly used treatments are corticosteroids, nonsteroidal anti-inflammatory drugs, hyaluronan and polysulfated glycosaminoglycan [2]. However, to date, none of these treatments halts the disease, let alone reverse it, so none of the current treatment modalities presents a durable solution for OA [4].

Regenerative medicine represents an interesting alternative for treating OA, since it has the potential to prevent further cartilage damage and even reverse the sustained damage [4–7]. Intra-articular use of native mesenchymal stem cells (MSCs) has shown promising, though modest, results for enhancing cartilage repair [4–8]. Moreover, the application of chondrogenic-induced MSCs combined with plasma in horses with OA has been shown to have a clinical advantage compared with using plasma alone or native MSCs combined with plasma [7,8]. In those two studies, allogeneic MSCs (from another animal than the case, but from the same species) instead of autologous MSCs were used, as allogeneic MSCs allow 'off-the-shelf' therapy. However, randomised, double-blinded, placebo-controlled studies to objectively investigate efficacy of allogeneic MSCs are currently lacking.

Therefore, the goal of this study was to assess the efficacy of a single intra-articular injection of equine allogeneic chondrogenic-induced MSCs combined with equine allogeneic plasma in an experimental model of metacarpophalangeal (MCP) OA. We hypothesised that this combination would have a superior effect on OA improvement compared with saline (0.9%).

Materials and methods

Study design and animals

Twelve healthy warmblood horses, three geldings and nine mares (median age 8.5 years), were enrolled in this blinded, controlled,

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[2]Both these authors contributed equally.

randomised and blocked study. The horses were free of visual lameness and presented no radiographic abnormalities of the MCP joints. The treatment was randomised using Randomizer_2 (2.1.0).xls. The horses were assigned to either the intervention group (n = 6) or to the control group (n = 6) per block of 2 horses (ratio 1:1).

Intervention and control product

Intervention product: The intervention consisted of a proprietary formulation of equine allogeneic chondrogenic-induced MSCs (ciMSCs) derived from peripheral blood (2×10^6 cells in 1 mL of Dulbecco's modified Eagle medium low glucose with 10% of dimethyl sulfoxide)[a] combined with equine allogeneic plasma (EAP) (1 mL plasma with 98×10^6 platelets/mL) (Arti-Cell® Forte)[b] . The ciMSCs and EAP were each stored in separate vials at −80°C until clinical application. Both donor horses (one for the ciMSCs and one for the EAP) were screened for 32 equine pathogens and collection of their blood was approved by the local ethics committee (approval number: EC_2012_001). These horses were not involved further in this study in any way. The IVP was produced according to Good Manufacturing Practice guidelines (BE/GMP/2015/082 and BE/GMP/2016/069) with a manufacturing authorisation (1868V) for veterinary medicinal cell-based products.

Control product: The control product consisted of a sterile saline solution (0.9% NaCl)[c] .

OA joint model and exercise programme

Osteoarthritis was surgically induced in the right MCP joint of all horses at the study start (Week 0) as previously described [9]. Briefly, the joint was arthroscopically assessed for the presence of pre-existing cartilage lesions. These lesions were documented but were not an exclusion criterion. In the right MCP joint, a dorsomedial P1 osteochondral (OC) fragment was created that remained attached to the dorsal joint capsule and the fragment bed was debrided with an arthroburr to decrease apposition between fragment and fracture bed. Next, a horizontal groove in the cartilage on the dorsal aspect of the medial condyle of the third metacarpal bone was created using the arthroburr. Skin incisions were closed and a bandage was applied.

All horses received a single dose of morphine (0.1 mg/kg btw i.v.) and antibiotics (sodium benzylpenicillin 10^7 iu i.v.) during surgery. No medication was administered post-operatively. Horses were box rested for 1 week after surgery, after which they were treadmill exercised for the remainder of the study period as previously described [9].

Treatment administration

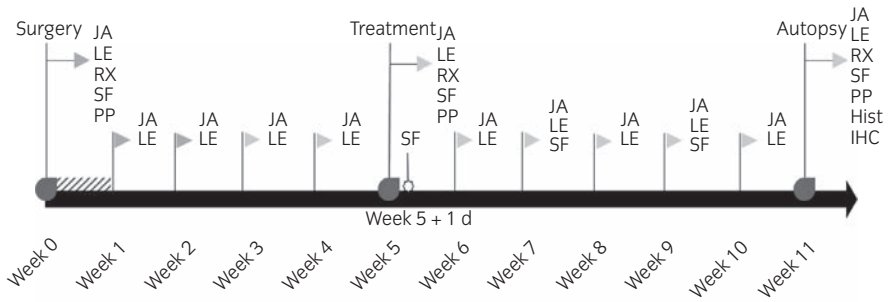
At Week 5, the intervention or control product was administered in the right MCP joint after sedation with detomidine hydrochloride (i.v., 20 μg/kg bwt). One vial of ciMSCs and one vial of EAP were thawed and drawn into one syringe, so a total volume of 2 mL was obtained. The same volume was used for the control product. Because of the nature of the intervention and control product (colour difference), this study was blinded by using separate personnel for clinical and laboratory examinations (investigators) and for administration of treatments (dispenser). One and the same investigator performed the clinical scorings throughout the study. Another investigator performed the histological scoring. For the macroscopic evaluation of the joint after euthanasia, a consensus score was derived after reaching an agreement between these two scoring investigators.

Efficacy outcome

Clinical and joint assessment: Horses underwent a daily general clinical examination and a weekly joint assessment throughout the entire study period (Fig 1). The joint assessment consisted of an evaluation of local temperature, joint effusion, pain on palpation, range of motion and measuring of the joint circumference using a measuring tape. An overview of the scoring system used for the joint assessment parameters can be found in Supplementary Item 1.

Lameness examination: A weekly visual and objective lameness examination was performed during the entire study period. The horses were examined on a treadmill, during lungeing on a soft surface, and in a straight line before and after distal forelimb flexion. Lameness was visually scored using the AAEP lameness scale [10]. The response to flexion was scored as follows: 0 = no response to flexion, 1 = mild response to flexion, 2 = moderate response to flexion and 3 = severe response to flexion. Objective evaluation was performed using an inertial sensor-based system (The Equinosis Q with Lameness Locator software)[d] [11,12]. A positive vector sum represented right front limb lameness and a negative one left front limb lameness. At Weeks 0, 5 and 11, a pressure plate analysis was performed using a combination of a 2-m pressure plate (RSscan 3D 2 m-system)[e] and a force plate (AMTI BP4602070RS-2K)[f] providing dynamic calibration of the pressure plate as previously described [13,14]. Symmetry indices were calculated and expressed as % symmetry (left/right × 100%).

Radiographic examination: Radiographs of both MCP joints including lateromedial, dorsopalmar and 45-degree dorsolateral–palmaromedial and dorsomedial–palmarolateral oblique projections were taken before and the



: Stall rest
JA: Joint assessment
LE: Lameness examination
RX: Radiographic examination
SF: Synovial fluid analysis
PP: Pressure plate analysis
Hist: Histology
IHC: Immunohistochemistry

Fig 1: A schematic presentation of the adhered time line with an overview of the different procedures performed at each time point.

day after surgery, and at Weeks 5 and 11. Radiographic changes were recorded.

Synovial fluid analysis: Synovial fluid samples were collected during surgery, the day of and after treatment and at Weeks 7, 9 and 11. Cytological evaluation was performed using a haematology analyser, and viscosity was assessed using following scoring system: 0 = watery, no string, 1 = tacky, string <0.5 cm, 2 = string 0.5–4 cm and 3 = string >4 cm. The following biomarkers were determined using commercial ELISA kits according to the manufacturers' instructions: IL-10[g], IL-1 receptor antagonist protein (IRAP)[h], PGE2[i], MMP-13[g], IL-6[j], serum amyloid A (SAA)[g], TNF-α[h], IFN-γ[h], hyaluronic acid (HA)[k], glycosaminoglycans (GAGs)[k,l] and TGF-β3[j].

Post-mortem examination: After all examinations were performed at Week 11, all horses were subjected to euthanasia using an i.v. injection with a combination product containing embutramide (T-61)[m].

Gross examination and histology—Both MCP joints were evaluated macroscopically for cartilage and synovial health according to the guidelines of the Osteoarthritis Research Society International (OARSI) [14] as described previously [9]. Cartilage was collected from both MCP joints from the area adjacent to the created fragment and at the level of the groove lesion. In addition, joint capsule and synovium were sampled. All samples were fixed in a 4% formaldehyde solution, embedded in paraffin, sectioned at 4 μm thickness and stained with haematoxylin and eosin. Cartilage repair and joint inflammation were evaluated using modified OARSI histological guidelines [15] as previously described [9]. Moreover, the presence of ectopic tissue was recorded. Cartilage samples were also stained with Alcian Blue to assess GAGs content through area % calculations (see Immunohistochemistry).

Immunohistochemistry—Immunohistochemistry was performed on the collected tissue samples to evaluate cartilage oligomeric matrix protein (COMP), collagen type II, Ki67 and caspase 3 expression. Tissue sections were stained with rabbit polyclonal anti-COMP (ab74524, 1:50)[g], anti-collagen II (ab34712, 1:50)[g], anti-caspase 3 (ab4051, 1:200)[g] and mouse monoclonal anti-Ki67 (M7240, 1:20)[n] respectively. Immunolabelling was achieved with a high-sensitive horseradish rabbit diaminobenzidine kit with blocking of endogenous peroxidase (Envision DAB+ kit)[o] in an autoimmunostainer (Cytomation S/N S38-7410-01)[m]. Positive staining was confirmed on microscopy, and the area percentages of three randomly photographed areas (at 200× magnification) were calculated per section with the use of LAS V4.1 software[p] [16].

Data analysis

All statistical analyses were performed using SAS statistical analysis software version 9.3[q] . The treatment groups were compared at baseline for sex using the Fisher's exact test and for age and fetlock circumference using the Wilcoxon rank-sum test.

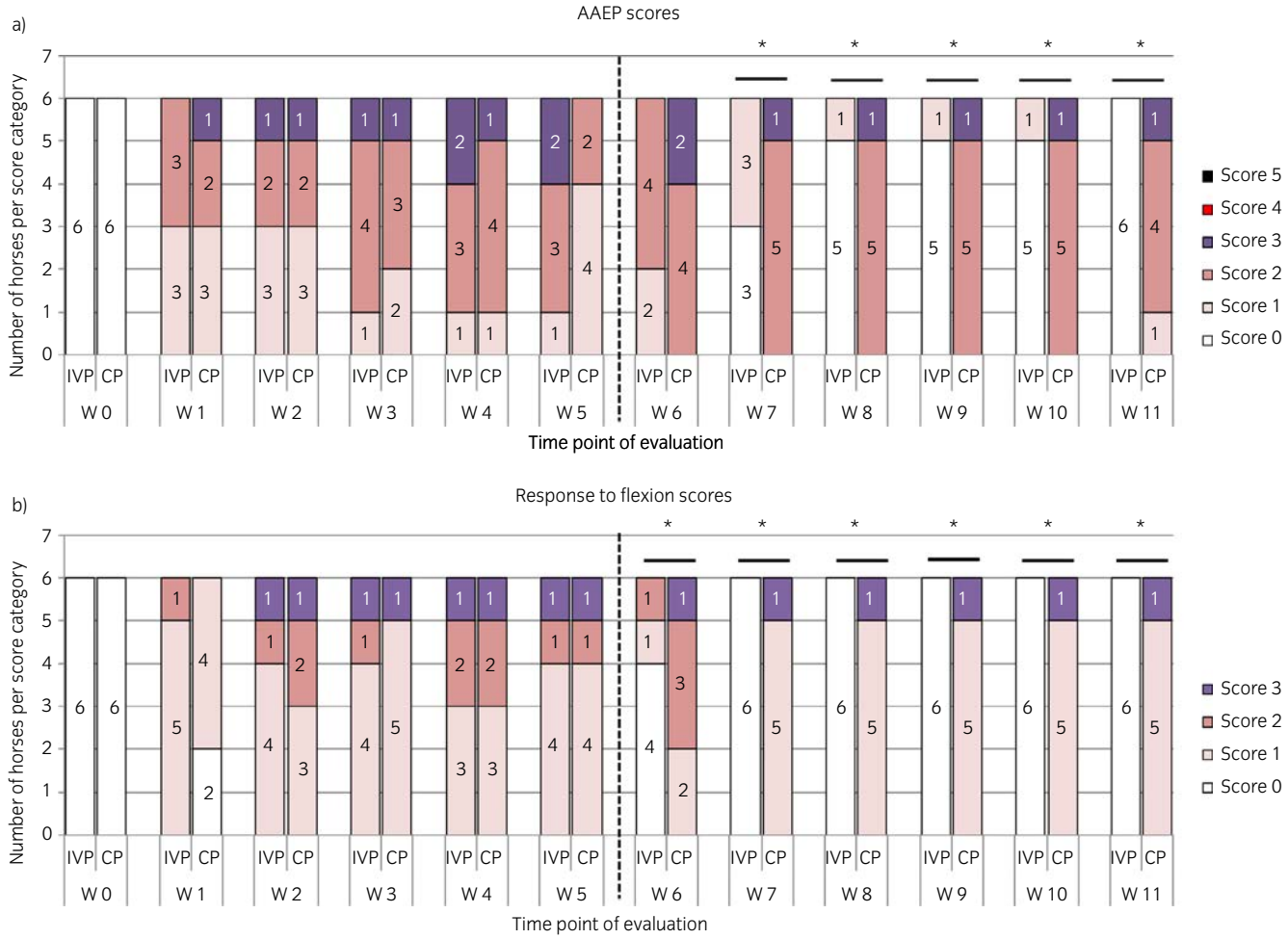


Fig 2: The evolution of the a) American Association of Equine Practitioners (AAEP) lameness scores and b) the response to flexion scores over the entire study period of 11 weeks for the horses treated with the placebo control product (CP) and the investigational veterinary product (IVP). The number of horses per score category is displayed per time point of evaluation. W, week. *Significant difference between the two treatment groups for that time point of evaluation with P<0.05.

Data of the joint assessment, visual lameness examination, response to flexion, viscosity of synovial fluid, macroscopic and histological evaluation were compared between the treatment groups with the Cochran–Mantel–Haenszel test. Data of the joint circumference measurements, inertial sensor analysis, pressure plate analysis, biomarker analysis, synovial fluid cell counts, Alcian blue stain and immunohistochemistry were compared between the treatment groups, using the Wilcoxon signed-rank test. A Fisher's exact test was performed to evaluate the data of the radiographic analysis (the presence or absence of radiographic changes) and the incidence of adverse events.

Since no detailed information on the different endpoints suing this treatment is available, an exact sample size could not be determined. Nevertheless, based on previous studies of our group using MSCs, a treatment success of 83% was expected, and a placebo effect of improvement of approximately 17% [7,8]. Based on a power of 80% and an alpha value of 0.05, this resulted in a sample size of 6 animals per treatment group. Moreover, this sample size has shown to generate statistical significant results in previous studies [5,17,18]. A larger sample size was not included due to ethical considerations.

Results

Animals

No statistically significant differences were found between the treatment groups at day 0 for sex, age, and MCP joint circumference. For six horses (three in each treatment group), mild cartilage changes were documented during arthroscopy for the right MCP joint consisting of a superficial wear line, partial erosion, minor irregularity or thinner cartilage spots. The synovium was normal in all horses.

Efficacy outcome

Lameness examination: There was no statistically significant difference in visual lameness scores or response to flexion between the two groups before treatment at Week 5. After treatment, AAEP scores were significantly lower with the intervention compared with the control from Week 7 onwards (P = 0.002; Fig 2a). In addition, response to flexion was also significantly lower with the intervention compared with the control from Week 6 onwards (P = 0.02 at Week 6, P = 0.001 from Weeks 7 to 11) (Fig 2b).

There was no significant difference in average vector sums between the treatment groups before treatment administration under any of the circumstances (Fig 3). At Weeks 9, 10 and 11, the vector sums on the treadmill were significantly lower in the intervention group compared with the control group (P = 0.02) (Fig 3a). In addition, average vector sums on a straight line after flexion were also significantly lower in the intervention group from Week 7 until Week 10 (P = 0.040 for Week 7 and P = 0.017 for weeks 8, 9 and 10). At Week 11, the average vector sums were lower with the intervention than with the control (P = 0.05 for Week 11) (Fig 3d). On the left circle and on a straight line before flexion, the average vector sums were lower after treatment in the intervention group compared with the control group, especially at Week 10 and 11, but these differences were not significant (Fig 3b, c). No statistically significant differences in symmetry indices were found at any time point between the treatment groups with the pressure plate analysis.

Clinical assessment, joint assessment and radiographic examination: No serious adverse events or suspected adverse drug reactions occurred during the study.

Before treatment, there were five horses with no increase in local temperature and one horse with a slight increase in both the treatment groups. All horses in the intervention group reached normal local temperature at the injection site from Week 6 until the end of study. In the control group, all horses reached normal local temperature the day after treatment until the end of study.

Before treatment, there were five horses with no pain on palpation and one horse with slight pain on palpation in both the treatment groups. All horses in both the treatment groups were without pain to palpation from the day after the treatment until the end of study.

After surgery, there was a limited range of motion in one horse in the intervention group at Week 2 and in one horse of the control group at

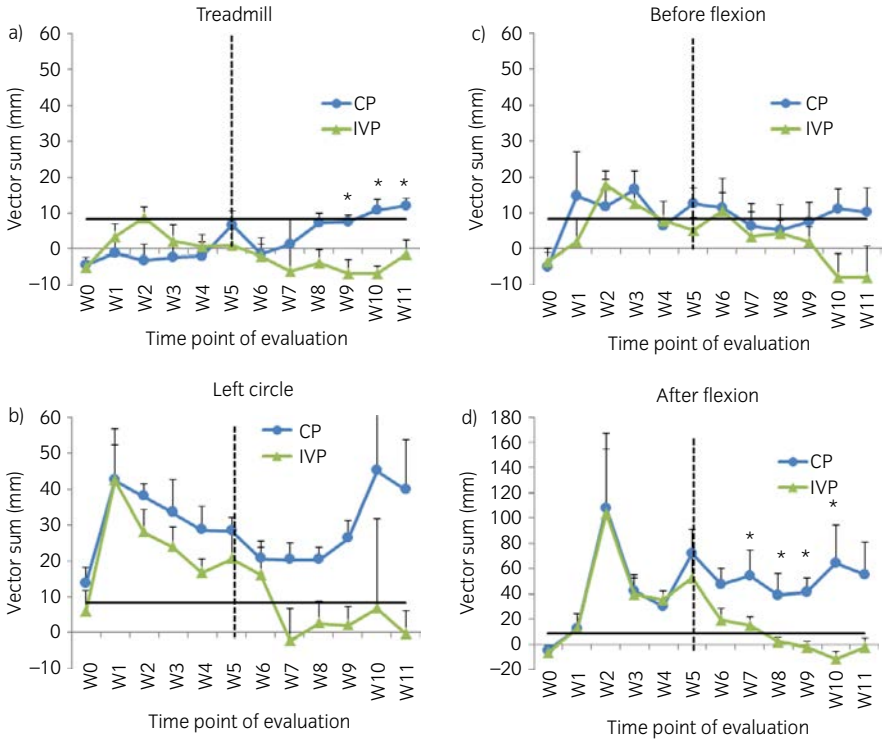


Fig 3: The evolution of the mean vector sums (+s.e.) measured with the inertial sensor-based system at the different time point of evaluation on a) treadmill, b) left circle, c) straight line before flexion and d) straight line after flexion. W, week. CP, control product. IVP, investigational veterinary product. *Significant difference between the two treatment groups for that time point of evaluation with P<0.05. The black horizontal bar indicates the threshold for lameness (i.e. 8.5 mm).

Week 3. No change in range of motion was found in any of the animals after Week 3.

There was no significant difference in joint effusion between the groups until Week 7. From Week 8 onwards, the joint effusion scores were significantly decreased with the intervention compared with the control (P = 0.005, P = 0.01, P = 0.005 and P = 0.01 for Weeks 8, 9 10 and 11 respectively) (Fig 4a). There was no significant difference in joint circumference between the treatment groups on any study day. The number of radiographic changes was not significantly different between the two treatment groups.

Synovial fluid analysis: A significantly higher viscosity score was seen in the intervention group at Week 9 (P = 0.02) and at Week 11 (P = 0.006) (Fig 4b). There was, however, no significant difference between the treatment groups in total white blood cells, lymphocytes, monocytes, granulocytes and total protein at any of the time points. In addition, no significant differences were seen in IL-10, PgE2, TGF-β3, HA, IRAP, IL-6, IFN-γ, TNF-α, SAA and MMP-13 concentrations. However, at Week 7, the GAGs concentration in the synovial fluid was significantly lower in the intervention group compared with the control group (mean concentration (±s.d.) of 19.9 μg/mL ± 5.6 vs. 44.0 ± 28.7 μg/mL respectively P = 0.04).

Post-mortem examination: Gross examination and histology—Significantly less wear lines were present in the intervention group compared with the control group on gross examination (median [range] wear line score of 0.5 [1] vs. 2 [2] for intervention and control respectively P = 0.02) (Fig 5a). In addition, synovitis was more prominent in the control group (median [range] of 1 [1]) than in the intervention group (median [range] score of 0 [2]), but the difference was not statistically significant (P = 0.061). Synovial hyperaemia however was significantly less pronounced in the intervention group than in the control group (median [range] score of 0 [1] vs. 1 [1] respectively P = 0.01) (Fig 5a). No statistically significant differences were found in erosions, extent of erosions, palmar arthrosis, covering of subchondral bone with fibrocartilage and synovial petechiation.

Histologically, there was no significant difference between the intervention and control groups in chondrocyte necrosis, cluster formation, fibrillation/fissuring, focal cell loss, cellular infiltration, vascularity, intimal hyperplasia, subintimal oedema or subintimal fibrosis. However, a significantly higher Alcian Blue uptake, an indirect measure of the amount of GAGs, was seen in the cartilage adjacent to the created OC fragment in the intervention compared with the control group (P = 0.02) (Fig 5b). No statistically significant difference was seen for the cartilage located at the groove. There was no presence of ectopic tissue in any animal.

Immunohistochemistry—The area % of COMP in the cartilage adjacent to the OC fragment was significantly higher in the intervention group compared with the control group (P = 0.02) (Fig 5b). The area % of COMP in the cartilage of the groove lesion was not statistically different between the two treatment groups. Collagen type II area % in the cartilage adjacent to the OC fragment and to the groove lesion was significantly higher in the intervention group than in the control group (P = 0.02) (Fig 5b). The area % of Ki67 and Caspase 3 were 0% in both the treatment groups.

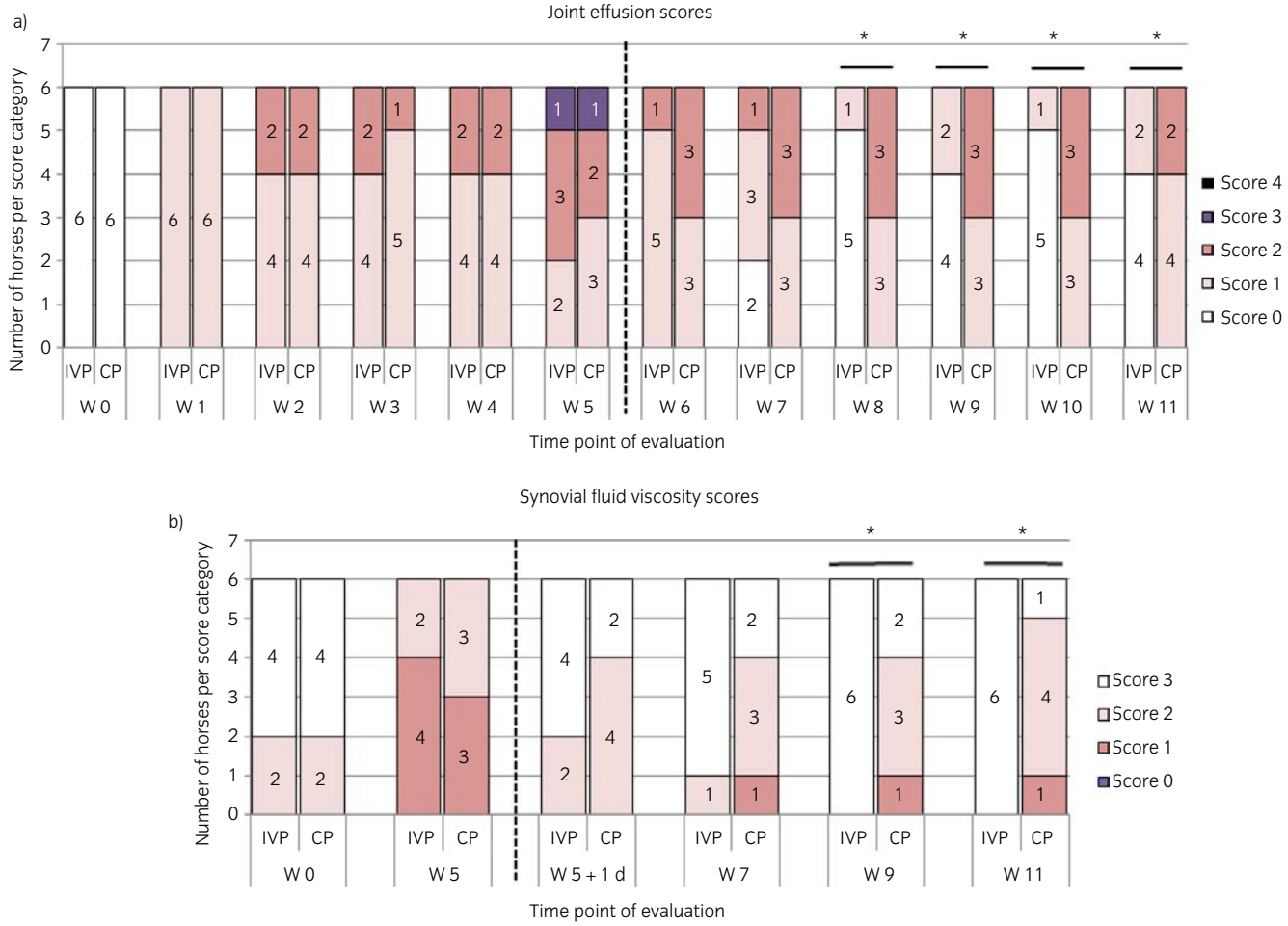


Fig 4: The evolution of the a) the joint effusion scores and b) the synovial fluid viscosity scores over the entire study period of 11 weeks for the placebo control product (CP)-treated horses and the investigational veterinary product (IVP)-treated horses. The number of horses per score category is displayed per time point of evaluation. W, week; d, day. *Significant difference between the two treatment groups for that time point of evaluation with P<0.05.

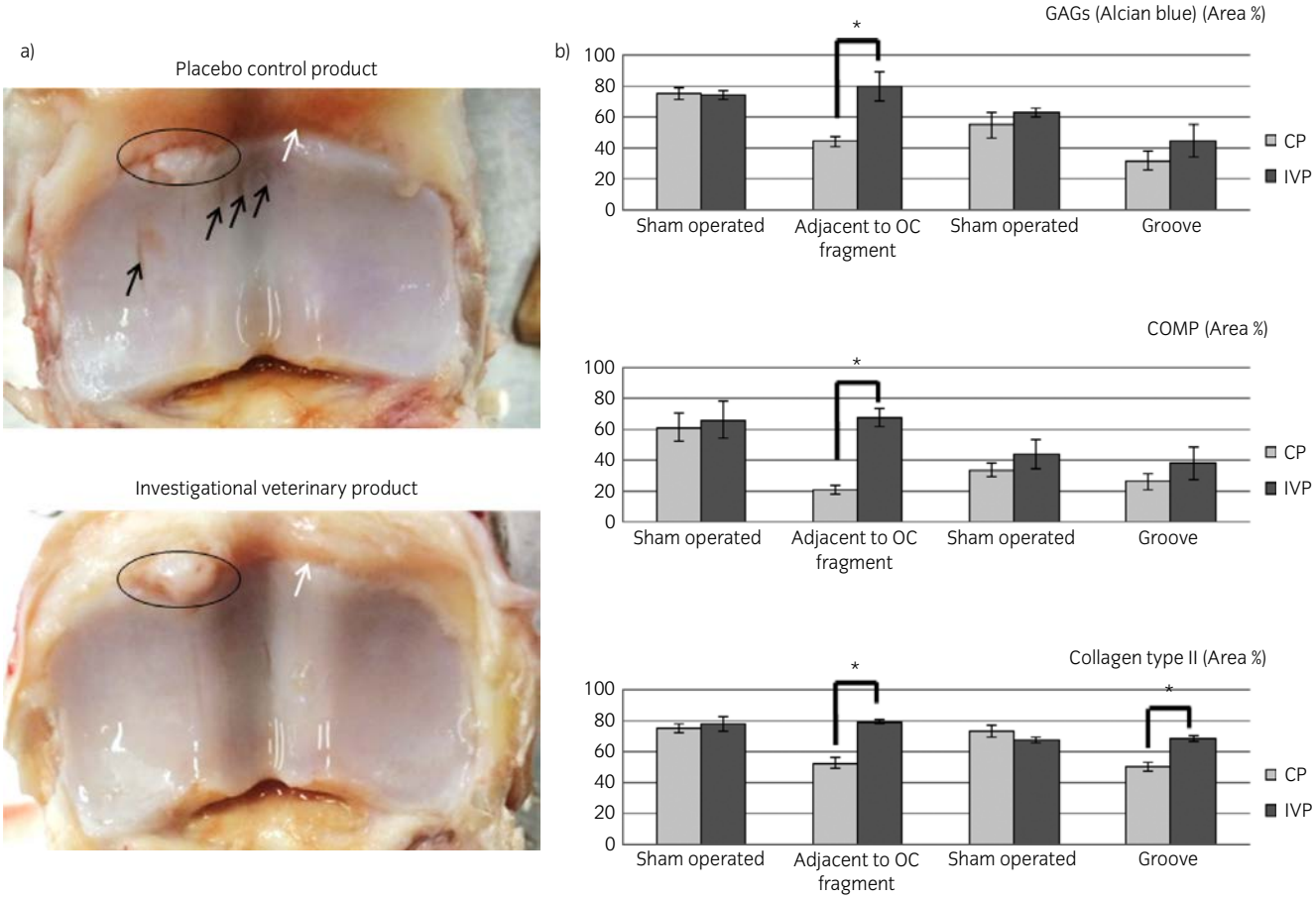


Fig 5: a) A representative example at gross examination of the proximal surface of the first phalanx of a horse treated with the placebo control product (CP) and a horse treated with the investigational veterinary product (IVP) (Week 11). Full thickness wear lines (indicated with the black arrows) are visible in the CP-treated joint, but not in the IVP joint. Synovial hyperaemia (indicated with the white arrow) was also more pronounced in the CP-treated joint compared with the IVP-treated joint. In both joints, the dorsomedial osteochondral (OC) fragment, which was still attached to the joint capsule, was clearly visible (encircled). b) The mean area percentages (+s.e.) of glycosaminoglycans (GAGs), cartilage oligomeric matrix protein (COMP) and collagen type II as seen during histological and immunohistochemical analysis of the articular cartilage sampled adjacent to the OC fragment and at the level of the groove lesion of the CP- and IVP-treated horses and of the equivalent areas in the healthy sham operated joints. *Significant difference between the treatment groups with $P<0.05$.

Discussion

After application of the OA model, horses clinically improved when they were treated with chondrogenic-induced MSCs combined with equine allogeneic plasma (= intervention) compared with when they were treated with saline (= control). Indeed, a significant reduction in visual and objective lameness was seen for the intervention group together with a reduced joint effusion and improved viscosity of the synovial fluid, suggesting symptom relieve by the intervention when compared with the control treatment. Post-mortem examination revealed a gross improvement of cartilage appearance, with a favourable local cartilage composition in the intervention group. Therefore, our results suggest an improvement of local cartilage health and metabolism [3]. This improvement was also suggested by the lower concentration of GAGs in the synovial fluid of the intervention group joints. Indeed, a higher concentration of GAGs in synovial fluid indicates a release of GAGs from diseased articular cartilage into the synovial fluid, as is seen in naturally occurring OA [19,20].

The pressure plate analysis did not reveal significant differences in the current study, probably because this analysis was performed at the end of all examinations and horses with OA generally display less lameness after warming up [21]. Moreover, the pressure plate analysis only allows evaluation of a limited number of steps while the animals are trotted in a straight line over the plate. In contrast, the evaluation of multiple strides using the inertial sensor-based system revealed a significant treatment effect under different circumstances.

Overall, the results observed in this study were more conclusive compared with those of previously reported studies on MSCs for articular cartilage repair. Wilke *et al.* [5] only found an initial improved cartilage healing after application of bone marrow-derived MSCs combined with a fibrin vehicle in induced cartilage lesions. Frisbie *et al.* [4] evaluated adipose-derived stromal vascular fraction and bone marrow-derived MSCs for treatment of OA, and reported a greater improvement during various evaluations with bone marrow-derived MSCs compared with placebo treatment and adipose-derived stromal vascular fraction cells, but the observed effects did not reach statistical significance. McIlwraith *et al.* [6] evaluated the use of bone marrow-derived MSCs as an addition to microfracture to augment healing of induced cartilage lesions, and reported higher quality repair tissue and a higher cumulative score during arthroscopic and gross evaluation with bone marrow-derived MSCs. However, no clinical improvement was seen in that study. Despite the short-term evaluation in the current study, significant effects of the intervention product were seen in the clinical, gross and histological examination. Differences between the current study and the previous studies mentioned could be related to differences in the stem cell product. In contrast to studies mentioned earlier [4–6], MSCs in the present study were chondrogenic-induced, which has been shown to result in better cartilage adherence in explant cultures [22]. In addition, a

previous study from our group has shown a higher return-to-work-rate when using chondrogenic-induced MSCs combined with plasma compared with native MSCs combined with plasma [8]. Moreover, the studies of Wilke *et al.* [5], Frisbie *et al.* [4] and McIlwraith *et al.* [6] used autologous stem cell preparations, while in the current study allogeneic MSCs were used. The use of an allogeneic product allowed high standardisation of the MSCs resulting in one uniform product for all horses in this study.

The intervention product we used had already been tested in an earlier target animal safety study [23]. Despite the cells being allogeneic, only a transient and mild local inflammatory response was seen in that study (mild increase in lameness, heat and joint effusion), which was not significantly different from the response after a saline injection. In the present study, again no serious adverse events or suspected adverse drug reactions were seen. Moreover, the joint effusion and AAEP scores were significantly lower in the intervention group from, respectively, 3 weeks and 2 weeks after treatment administration onwards. A possible explanation of the lack of on inflammatory response to the ciMSCs used in the present study is that these cells are negative for MHC class II molecules and have a very low MHC class I expression [7]. These characteristics could possibly allow the cells to be immune evasive, since it is known that both MHC I and MHC II expression on MSCs can induce an immune response in MHC-mismatched individuals [24,25].

In the current study, an experimental model was used to obtain standardised circumstances. However, only the short-term effects of the intervention product were tested (6 weeks), whereas naturally occurring OA inherently presents more variability in the stage of the disease, the speed of progression and the innate healing response of the individual patient. Moreover, multiple statistical tests were performed, increasing the risk of type 1 error. Therefore, the results of this experimental study should be confirmed in a field trail on a large number of animals with naturally occurring disease.

Conclusion

Equine allogeneic ciMSCs combined with EAP resulted in a significantly reduced lameness and joint effusion compared with a placebo treatment after a single intra-articular injection in MCP joints with experimentally induced OA. In addition, a significantly improved synovial fluid viscosity, reduced number and/or severity of wear lines, and a decreased synovial hyperaemia was noted. Equine allogeneic ciMSCs combined with EAP also improved local cartilage composition by significantly increasing the amount of GAGs, COMP and collagen type II compared with saline-treated control joints. Therefore, equine allogeneic ciMSCs combined with EAP could potentially be a promising treatment of OA in horses.

Authors' declaration of interests

J. Spaas declares competing financial interests as shareholder in Global Stem cell Technology (GST) NV. S. Broeckx, J. Spaas and L. Van Hecke are all employed by GST. S. Broeckx and J. Spaas are inventors of several pending patents owned by GST (BE2012/0656; WO2014053418A9; WO2014053420A1; PCT/EP2013/075782). The other authors declare no competing interests. The content of this manuscript contains the product Arti-Cell® Forte owned by GST.

Ethical animal research

This study and its protocol were approved by the local ethics committee of Global Stem cell technology (approval number EC_2015_002; Permit Number: LA1700607). Collection of the donor blood was approved by the local ethics committee (approval number: EC_2012_001).

Sources of funding

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Acknowledgements

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Authorship

J. Spaas, S. Broeckx, A. Martens, F. Pille, M. Dumoulin, M. Oosterlinck, L. Duchateau, A. Bertone, F. Pille and K. Chiers conceived and designed the experiments. A. Martens performed all surgeries mentioned in the study. S. Broeckx, J. Spaas, F. Pille, L. Van Brantegem, K. Chiers, M. Dumoulin, A. Bertone, H. Hussein and M. Oosterlinck performed the data collection and implementation of the experiments. L. Duchateau performed the statistical analysis. S. Broeckx, J. Spaas, L. Van Hecke and L. Duchateau interpreted the data. S. Broeckx, L. Van Hecke and J. Spaas drafted the manuscript. All authors and approved reviewed the manuscript for submission.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Summary in Spanish.
Supplementary Item 1: Clinical scoring system.

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Regenerative Therapies for Equine Degenerative Joint Disease: A Preliminary Study

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Abstract

Degenerative joint disease (DJD) is a major cause of reduced athletic function and retirement in equine performers. For this reason, regenerative therapies for DJD have gained increasing interest. Platelet-rich plasma (PRP) and mesenchymal stem cells (MSCs) were isolated from a 6-year-old donor horse. MSCs were either used in their native state or after chondrogenic induction. In an initial study, 20 horses with naturally occurring DJD in the fetlock joint were divided in 4 groups and injected with the following: **1**) PRP; **2**) MSCs; **3**) MSCs and PRP; or **4**) chondrogenic induced MSCs and PRP. The horses were then evaluated by means of a clinical scoring system after 6 weeks (T_1), 12 weeks (T_2), 6 months (T_3) and 12 months (T_4) post injection. In a second study, 30 horses with the same medical background were randomly assigned to one of the two combination therapies and evaluated at T_1. The protein expression profile of native MSCs was found to be negative for major histocompatibility (MHC) II and p63, low in MHC I and positive for Ki67, collagen type II (Col II) and Vimentin. Chondrogenic induction resulted in increased mRNA expression of aggrecan, Col II and cartilage oligomeric matrix protein (COMP) as well as in increased protein expression of p63 and glycosaminoglycan, but in decreased protein expression of Ki67. The combined use of PRP and MSCs significantly improved the functionality and sustainability of damaged joints from 6 weeks until 12 months after treatment, compared to PRP treatment alone. The highest short-term clinical evolution scores were obtained with chondrogenic induced MSCs and PRP. This study reports successful *in vitro* chondrogenic induction of equine MSCs. *In vivo* application of (induced) MSCs together with PRP in horses suffering from DJD in the fetlock joint resulted in a significant clinical improvement until 12 months after treatment.

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Introduction

Degenerative joint disease (DJD) is a major cause of reduced athletic function and retirement in equine performers [1–3]. Medical treatment for DJD may include anti-inflammatory and analgesic drugs to reduce inflammation and pain, and so-called disease-modifying drugs such as glucosamine, chondroitin sulphate or hyaluronic acid [4–6]. In the case of severe cartilage and bone degeneration, the use of articular cartilage curettage, osteophyte removal or even arthrodesis could be suitable [4,7]. Nevertheless, the aforementioned therapies are merely aimed at alleviating the symptoms or enhancing clinical recovery, without inducing an actual regeneration of the affected joint.

The field of equine regenerative medicine is drawing increasing attention in the scientific community for its treatment strategies of joint pathologies. Equine mesenchymal stem cells (MSCs) are of specific therapeutic interest as they can differentiate *in vitro* towards cells with a hyaline-like cartilage morphology and produce cartilage-specific components such as collagen type II and glycosaminoglycans [8–10]. Moreover, horses may serve as a valuable large animal model for the evaluation of new human therapies concerning *in vivo* efficiency and safety, due to interspecies similarities in tendon structure [11,12] as well as thickness of the non-calcified cartilage of the stifle joint [13]. Therefore, the evaluation of new treatments for musculoskeletal injuries in horses may be of broad clinical benefit for both equine and human medicine.

Other than a single case report with a positive clinical outcome of naturally occurring DJD after MSC therapy [14], the few available placebo-controlled studies in horses consist of experimentally induced cartilage lesions [15–17], not entirely resembling the clinically observed pathology. Importantly, the micro-environ-

ment - or niche - in degenerated cartilage might not provide the correct signals for MSC differentiation or alternatively, may even negatively influence their viability. Therefore, a priori chondrogenic induction of MSCs may improve the clinical outcome. In fact, this approach of provoking tenogenic induction has been used in the past to treat different equine tendon lesions, with promising clinical results [18,19]. Therefore, a principal aim of this study was to evaluate the clinical effects of a combined therapy for the treatment of equine DJD, using either native MSCs plus platelet-rich plasma (PRP) or chondrogenic induced MSCs plus PRP. This approach was compared to the more conventional regenerative therapies based on the use of PRP or native MSCs alone, which have been shown to be clinically safe [18–26]. This study also sought to compare the therapeutic efficacies of chondrogenically-induced MSCs (plus PRP) to native MSCs (plus PRP).

Allogenic peripheral blood (PB) from one donor horse was used as the source of MSCs, since it has been previously reported that PB MSCs also have the capacity to produce cartilage *in vitro* [10,27]. Moreover, the same donor horse could be used to produce PRP, thus substantially increasing the standardization of the sample production and the comparability between the different treatment groups: **1**) PRP alone (n = 5); **2**) native MSCs alone (n = 5); **3**) native MSCs and PRP (n = 5); and **4**) chondrogenic induced MSCs and PRP (n = 5). Chondrogenic induction was assessed *in vitro* by immunocytochemistry and real-time RT-PCR analysis. A clinical scoring system was established in order to enable two non-blinded independent veterinarians to give their professional assessment of the clinical status of the injured joint. At different time points after treatment (6 weeks, 12 weeks, 6 months and 12 months), scores were given to all the patients.

Materials and Methods

This study was carried out in strict accordance with the recommendations of the Animal Welfare Department of the Belgian Federal Public Service of Health. The protocol was approved by the Committee on the Ethics of Animal Experiments 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 [19]. 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 buffy coat 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). The further isolation and characterization was performed as previously described [10].

Table 1. TaqMan gene expression assays used for real-time RT-PCR.

Target gene	Assay ID
Aggrecan	Ec03469667_m1
Collagen II	Ec03467386_g1
COMP	Ec03468079_g1
GAPDH	Ec03210916_gH

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Table 2. All the patients were clinically assessed for joint effusion, response to flexion test and lameness according to the American Association of Equine Practitioners (AAEP).

	Score	Clinical implication
Joint effusion	0	No swelling
	1	Moderate swelling
	2	Severe swelling
Flexion test	0	No flexion response
	1	Mild flexion response
	2	Moderate flexion response
	3	Severe flexion response
AAEP grading	0	No lameness
	1	Lameness not consistently regardless circumstances
	2	Lameness consistently under certain circumstances
	3	Lameness consistently observable on a straight line
	4	Obvious lameness: marked nodding or shortened stride
	5	Minimal weight bearing lameness in motion or at rest

Because the importance of each parameter was correlated with its impact, the sum of these 3 parameters was reckoned as the overall clinical severity score (0 to 10).

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After that, 20×10^6 peripheral blood mononuclear cells (PBMCs) were seeded per T_{75} flask in 3 flasks and expanded in culture medium consisting of low glucose (LG) DMEM, 20% foetal calf serum (FCS) and 1% antibiotics-antimycotics (AB/AM) [10]. The medium was refreshed twice a week and the cells were maintained at 37°C and 5% CO_2. At 60% confluency, the cells were trypsinized with 0.25% trypsin-EDTA and subcultured until passage 3, at which time cells were characterized as previously described [10] before seeding them at 6.7×10^3 MSCs/cm^2 in T_{75} flasks for expansion, or chondrogenic induction. Chondrogenic induction medium consisted of DMEM LG, 20% FCS, 1% AB/AM and cartilage-specific growth factors, similar to a previous report by Jonitz [28]. 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 (Arti-Cell® and Arti-Cell® Plus respectively).

Preparation of Platelet-rich Plasma (PRP)

In total, 300 ml of peripheral blood was taken in a citrate phosphate dextrose adenine-1 (CPDA-1) single blood bag (Terumo®) for platelet-rich plasma (PRP) preparation. From this donor horse, 30 samples of 1 ml PRP were prepared as previously described by our group [18,19]. Each sample contained approximately 200×10^6 platelets and was frozen and stored at −80°C before clinical application.

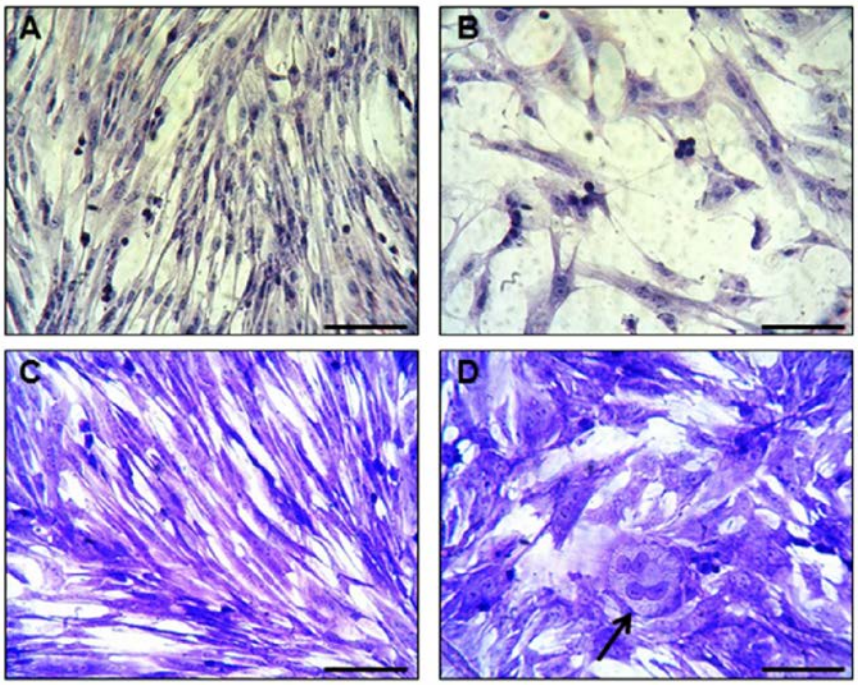

Figure 1. Representative images of peripheral blood (PB)-derived mesenchymal stem cells (MSCs) in their undifferentiated state (A & C) and chondrogenic induced (B & D) after Hematoxylin (A & B) and Crystal Violet (C & D) stainings. The typical chondrogenic morphology and lacune formation (black arrow) can be noticed after induction. Scale bars represent 50 µm.
doi:10.1371/journal.pone.0085917.g001

Cytological Staining

Hematoxylin (HE), Crystal Violet (CV), Alcian Blue (AB) and Safranin O (SO) staining (all from Sigma) were performed on MSCs and chondrogenic-induced MSCs, as indicated by the manufacturer. Both HE and CV staining were carried out, in order to visualize the cell morphology and cellular organization. Furthermore, AB and SO staining were performed to give an indication of the presence of acid polysaccharides, such as glycosaminoglycans in cartilage-like structures.

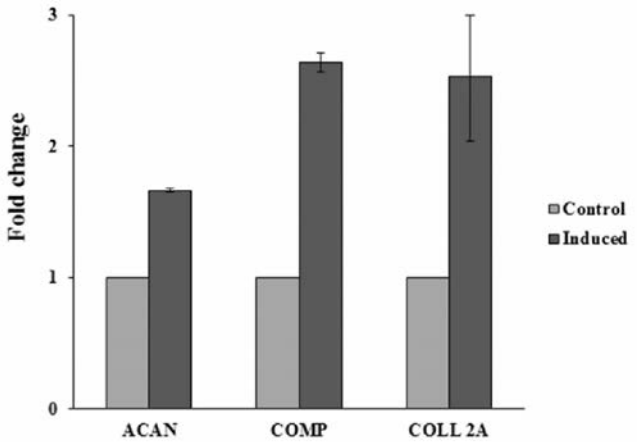

Figure 2. Results of RT-PCR for the gene expression of collagen (Col) type II, aggrecan and cartilage oligomeric matrix protein (COMP) in the native MSCs (Ctrl) and chondrogenic induced MSCs (Ind). Values are given as the mean of three measurements ± SEM.
doi:10.1371/journal.pone.0085917.g002

Immunocytochemistry

Immunocytochemistry was performed to evaluate the expression of collagen type II (Col II), Ki67 (proliferation marker), p63 (tumor suppression gene) and vimentin (mesenchymal cell marker) on native MSCs and chondrogenic-induced MSCs in adhesive tissue culture plates and after trypsinization and cytospin preparation at 700 rpm for 4 minutes. Cells were fixed for 10 minutes with 4% PF and permeabilized for 2 minutes with 0.1% Triton X at room temperature. Subsequently, cells were incubated with hydrogen peroxide (0.03%) for 5 minutes at room temperature and after washing with PBS, incubated for 30 minutes at room temperature with the primary rabbit IgG polyclonal antibodies recognizing: Col IIA1 (1:50), Ki67 (1:200) and p63 (1:100) and mouse IgG_1 monoclonal anti-vimentin (1:100) (all from Abcam). After washing with PBS, secondary ready-to-use goat anti-mouse and anti-rabbit peroxidase (PO)-linked antibodies (Dako) were added and incubated for 30 minutes at room temperature. Finally, 3,3′-diaminobenzidine (DAB) was added for 5 minutes and a counter staining with hematoxylin was performed to visualize the surrounding cells. As controls, identical staining was performed on undifferentiated MSCs and background staining was assessed by using the proper isotype-specific mouse monoclonal or rabbit polyclonal antibody. All isotypes were matched to the immunoglobulin subtype and used at the same protein concentration as the corresponding antibodies. Wherever appropriate, equine tendon or skin tissue sections were used as negative controls.

Flow Cytometry

To characterize the MSCs immunophenotypically, the expression of several stem cell markers was evaluated by flow cytometry, as previously described [10]. For the present study, we evaluated the expression of the typical rejection proteins, major histocom-

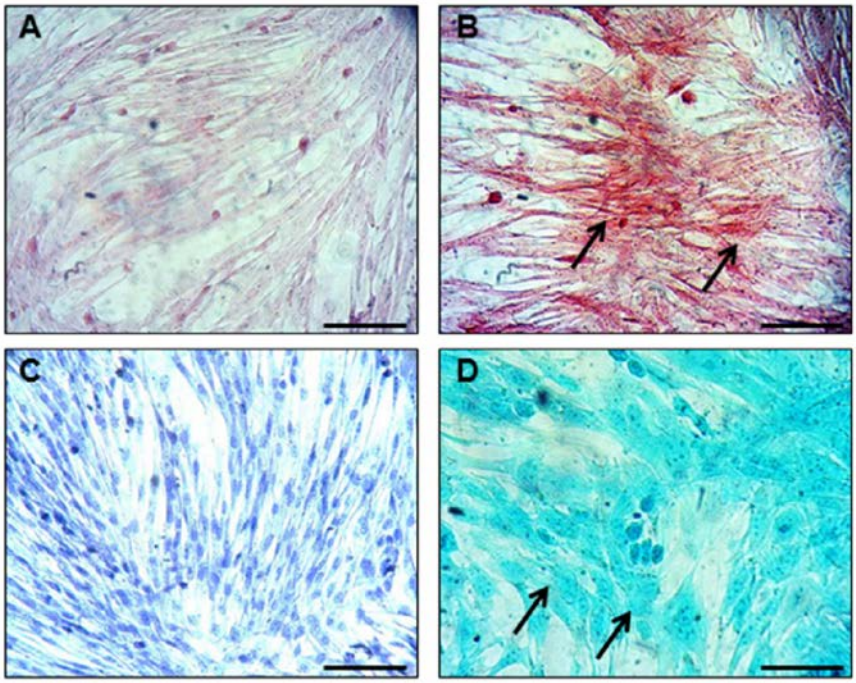

Figure 3. Representative images of peripheral blood (PB)-derived mesenchymal stem cells (MSCs) in their undifferentiated state (A & C) and chondrogenic induced (B & D) after Alcian Blue (A & B) and Safranin O (C & D) stainings. Glycosaminoglycan production (black arrows) can be noticed after induction. Scale bars represent 50 μm.
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patibility (MHC) class I and II on native and chondrogenic induced MSCs. Per series, 400'000 cells were used and labeled with the following primary antibodies: mouse anti-horse MHC class I IgG_{2a} (Washington State University, 1:50) and mouse anti-horse MHC class II IgG_1 (Abd Serotec, 1:50). Cells were incubated with the primary antibodies for 15 minutes on ice in the dark and washed twice in washing buffer, consisting of DMEM with 1% bovine serum albumin (BSA). A secondary rabbit anti-mouse-FITC (Abcam, 1:100) antibody was used to identify positive cells after 15 minutes of incubation on ice in the dark. Finally, all cells were washed three times in washing buffer and at least 10'000 cells were evaluated using a fluorescence activated cell sorter (FACS). All analyses were based on (i) autofluorescence and (ii) control cells incubated with isotype-specific IgG's, in order to establish the background signal. All isotypes were matched to the immunoglobulin subtype and used at the same protein concentration as the corresponding antibodies. As positive controls, PBMCs were used to confirm MHC cross-reactivity.

Gene Expression Analysis by Real-time RT-PCR

Equine MSCs in passage 3 were seeded in T_{25} flasks at a density of 8'000 MSCs/cm^2 with expansion medium or chondrogenic induction medium for 30 hours. After treatment, cells were lysed in 2 ml of Trizol (Invitrogen) and the lysate was separated into aqueous and organic phases by chloroform separation (300 μl, Sigma-Aldrich). The aqueous phase was recovered after centrifugation and total RNA was precipitated by using equal volumes of isopropanol. The precipitate was washed with 75% EtOH once and then solubilized with 25 μl of RNAse free water and quantified on the Nanodrop Lite (Fisher Scientific) before reverse transcribing 1 μg of RNA, using the TaqMan Reverse Transcription Reagents Kit (Life Technologies). Gene expression analysis was performed in triplicate (30 ng of cDNA in each reaction) with TaqMan Gene Expression Assays (Life Technologies) (Table 1) on the CFX96 Real-Time PCR System (Biorad). Values were normalized to GAPDH mRNA as internal control and presented as fold change, compared to native MSCs (i.e. in expansion medium), using the comparative CT method ($=2-\Delta\Delta CT$ method).

Patient Inclusion Criteria

For a first study, 20 acceptor horses were selected based on their injuries. To be included in this study, clinical lameness had to be present in a mild to moderate form for at least 3 months. Moreover, the observed locomotory disorder had to be attributable to fetlock (metacarpophalangeal or metatarsophalangeal) joint osteoarthritis. In this regard, the source of the lameness was confirmed by both local analgesia and a positive flexion test for all the patients. In all included horses, the lameness was exacerbated by a flexion test of the fetlock joint, and was abolished by intra-articular administration of a local anaesthetic solution. In the present study, 5 ml of 0.5% Mepivacaine Hydrochloride (Meaverin Actavis®) 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 fetlock joint had to be noticeable in the form of osteophytes and/or cartilage defects. For a second study (comparing 2 combination treatments), 30 horses were selected using the same inclusion criteria. 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

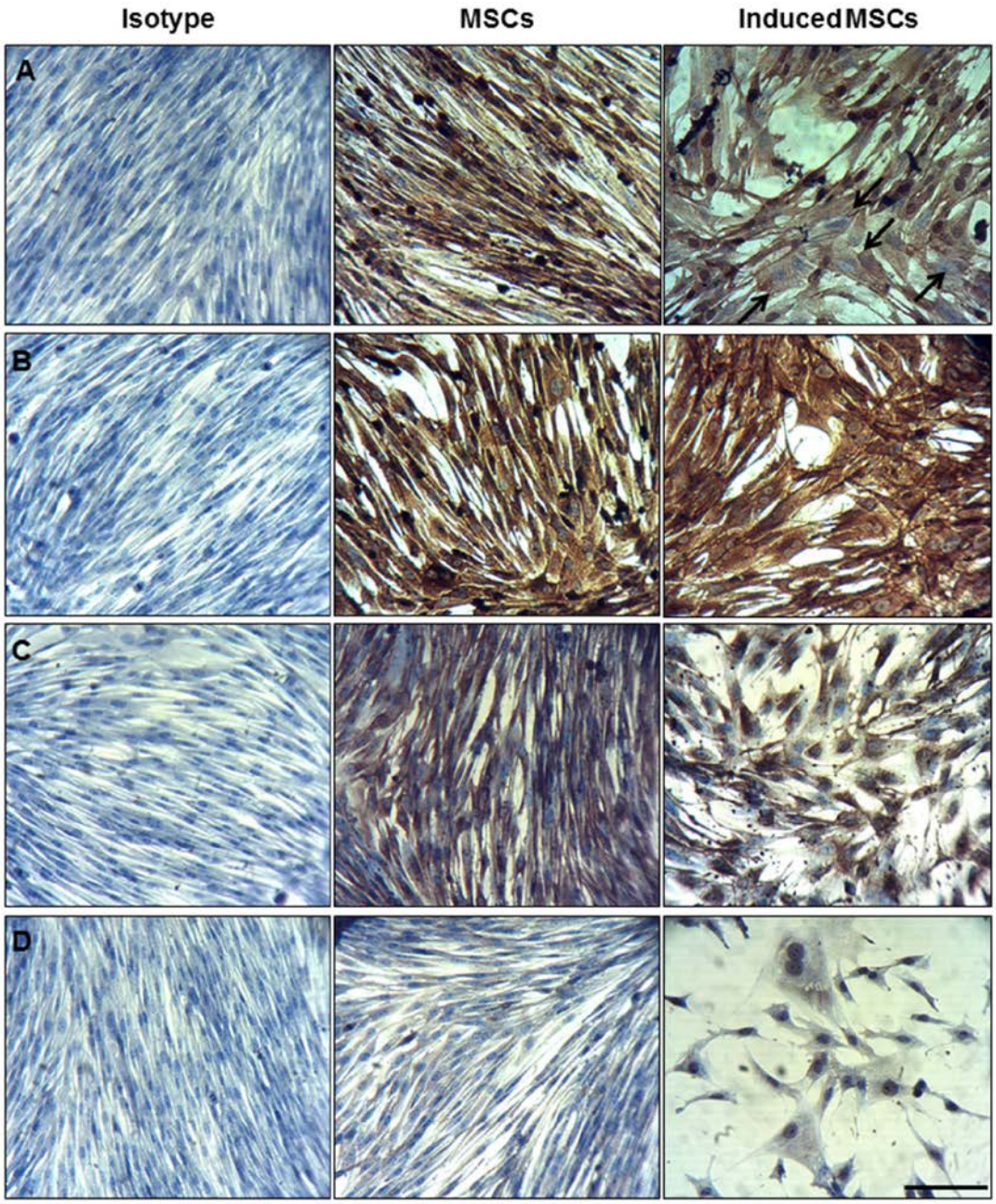

Figure 4. Immunocytochemistry on adhesive mesenchymal stem cells (MSCs) using Ki67 (A), collagen (Col) type II (B), vimentin (C) and p63 (D). Native MSCs were negative for p63 and positive for Ki67, Col II and vimentin, whereas chondrogenic induced MSCs were positive for p63, Col II and vimentin with a decreased signal for Ki67 (arrows = negative nuclei). The relevant isotype controls were negative. Scale bar represents 50 μm.
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that exposure of MSCs to high concentrations of anaesthetics negatively influences cell viability [29]. 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. In the first study, horses were randomly assigned to PRP, native MSCs, native MSCs and PRP (Combination 1), or chondrogenic-induced MSCs and PRP (Combination 2) treatment. In the second study, horses were randomly assigned to one of the two combination therapies. 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 monitored for 1 week by means of a daily examination of the injected joint and by observing the occurrence of possible adverse effects or hypersensitivity reactions (wheal formation, sweating, strong respirations or even fever). Subsequently, the joints were evaluated at approximately 6 weeks (T_1), 12 weeks (T_2), 6 months (T_3) and 12 months (T_4) post injection through clinical evaluation by 2 independent veterinarians for all horses. In the second study, horses were randomly assigned to one of the two combination therapies and evaluated at T_1. The ethical committee approved the experimental design (EC_2013_001).

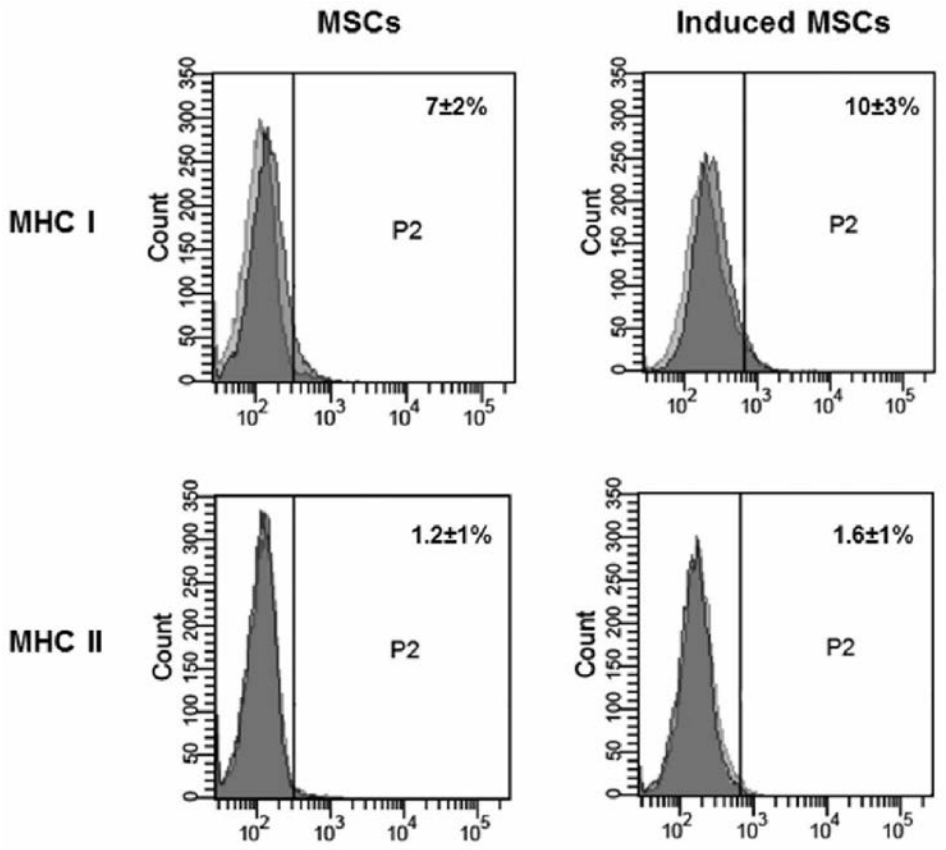

Figure 5. Flow cytometry confirmed a low expression of major histocompatibility complex (MHC) class I and no expression of MHC class II on the native MSCs and chondrogenic induced MSCs. The light and dark grey histograms represent the relevant isotype control staining and marker antibody staining, respectively with the corresponding percentage of mean positive cells ± SEM.
doi:10.1371/journal.pone.0085917.g005

Clinical Scoring System

In order to evaluate the severity of the clinical condition, the following parameters were graded by the same veterinarians at the aforementioned time points (T_{0-4}): clinical lameness from 0 to 5 (0 = no lameness and 5 = minimal weight bearing lameness) according to the American Association of Equine Practitioners (AAEP), response to flexion test from 0 to 3 (0 = no flexion response and 3 = severe flexion response) and fetlock joint effusion from 0 to 2 (0 = no swelling and 2 = severe swelling). Because the importance of each parameter was correlated with its impact, the sum of these 3 parameters was reckoned as the overall clinical severity score (0 to 10), with 0 corresponding to clinical soundness. A detailed overview of the different scores can be found in Table 2.

All the horses in this study showed initially a mild to moderate lameness (1–2 out of 5), mild to moderate response to flexion test (1–2 out of 3) and moderate to severe joint effusion (1–2 out of 2). As a result, all horses had a very similar initial clinical score of 4–5 out of 10. Progress was scored relative to before the treatment. Since none of the patients worsened, all the scores were greater than zero and translated in a positive evolution score ranging from 0 to 5:0 = severity score of 5 out of 10; 1 = severity score of 4 out of 10; 2 = severity score of 3 out of 10; 3 = severity score of 2 out of 10; 4 = severity score of 1 out of 10; and 5 = return to clinical soundness or severity score of 0 out of 10. Severity scores were translated to evolution scores for easier interpretation of the data and a positive trend would therefore indicate a clinical improvement. Statistical analysis was performed based upon the clinical evolution scores.

Statistical Analysis

For data analysis in study 1, the average of the evolution scores at 6 and 12 weeks represented the early evolution score, and the average of the evolution scores at 6 and 12 months represented the late evolution score. The early and late evolution scores are compared between the group receiving both MSCs (either native or induced) and PRP and the group receiving only MSCs on the one hand or receiving only PRP on the other hand, using the Wilcoxon signed rank sum test at the 5% significance level. Furthermore, within the combined treatment (study 2), the chondrogenic-induced MSCs are compared with native MSCs only for the earliest evolution score (i.e. at 6 weeks) equally using the Wilcoxon signed rank sum test at the 5% significance level.

Results

Isolation 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 [10], with the addition of several markers.

Characterization and Chondrogenic Induction of MSCs

To initially characterize MSCs and confirm chondrogenic induction, we analyzed cell morphology by light microscopy utilizing Hematoxylin and Crystal Violet staining. Biochemical induction was analyzed by measuring gene and protein expression

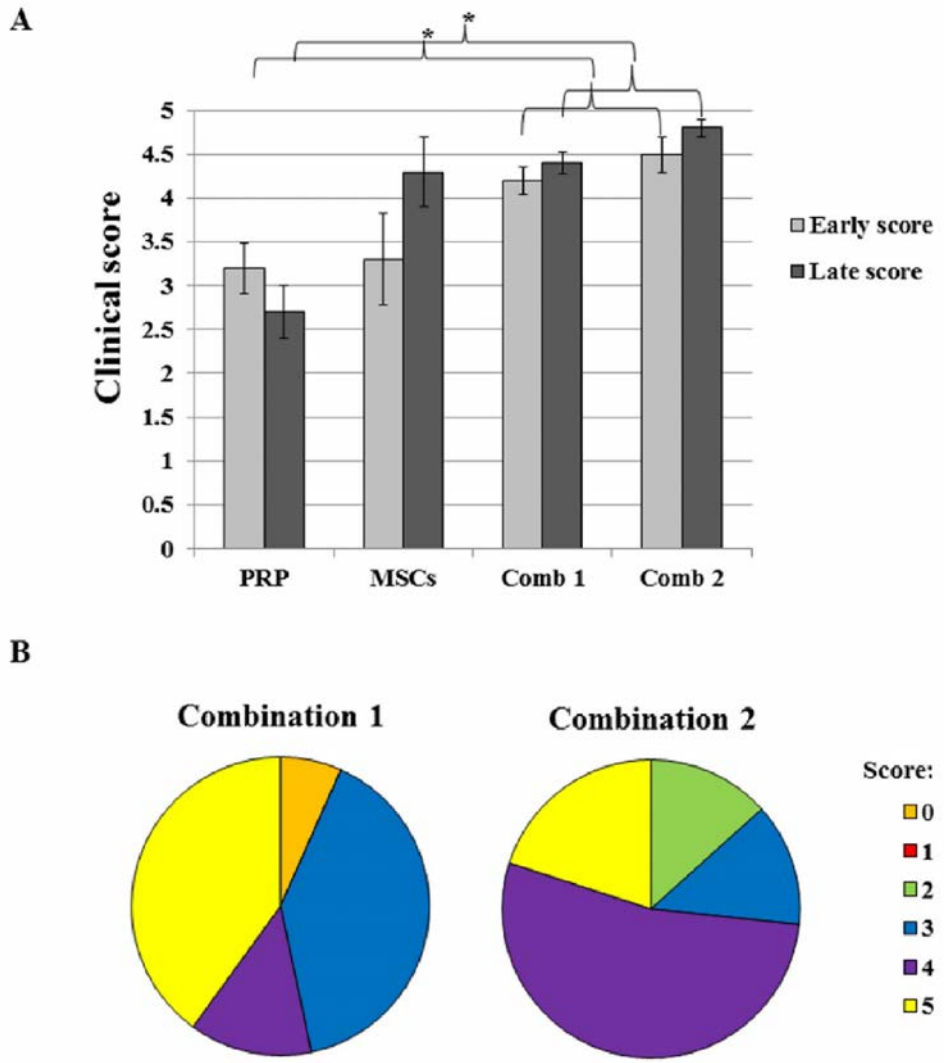

Figure 6. Clinical evolution scores of the different treatments at different time points in the first study (A). Values are given as the mean ± SEM. Diagrams represent the clinical evolution scores of 30 horses treated with native mesenchymal stem cells (MSCs) and PRP (Combination 1, n = 15) or chondrogenic induced MSCs and PRP (Combination 2, n = 15) in the second study (**B**).
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of selected cell markers (glycosaminoglycan production, collagen type II (Col II), Ki67 p63, vimentin, major histocompatibility complex, aggrecan, cartilage oligomeric matrix protein) providing insight into the degree of chondrogenic-induction by real-time RT-PCR, Alcian Blue staining, Safranin O staining, immunocytochemistry and flow cytometry.

Light microscopic analysis in conjunction with HE and Crystal Violet staining showed that native MSCs (Figure 1A & C) had a stellate/spindle-shaped morphology and displayed a propensity to grow in colonies, whereas MSCs induced into the chondrogenic lineage (Figure 1B & D) showed a more rectangular morphology. In addition, a few chondrocyte-like cells in lacune-like structures could be noticed after 3 days of culturing in the chondrogenic-inducing medium (Figure 1D). Gene expression analysis confirmed the switch towards a chondrogenic phenotype, exhibiting increases in the levels of Col II, aggrecan (ACAN) and cartilage oligomeric matrix protein (COMP) in induced MSCs, compared to native MSCs (Figure 2). Histological staining of the cells with both Alcian Blue and Safranin O confirmed the production of glycosaminoglycans in the chondrogenic-induced group (Figure 3B & D), whereas undifferentiated MSCs stained negative (Figure 3A & C).

Immunocytochemistry in adhesion (Figure 4) as well as after trypsinization and cytospin preparation (Figure S1) revealed that most of the nuclei in the native MSC group were positive for the proliferation marker Ki67, whereas noticeably less nuclei stained positively in the chondrogenic-induced group (Figure 4A, Figure S1A). Moreover, native MSCs and chondrogenic-induced MSCs were both positive for Col II (Figure 4B, Figure S1B). Adhesive culture and cytospin analysis further indicated that native and chondrogenic-induced MSCs were immunoreactive for vimentin (Figure 4C, Figure S1C), while p63 (Figure 4D, Figure S1D), which is a member of the p53 tumor suppressor gene family, was only detectable in chondrogenic-induced MSCs. Isotype (Figure 4, Figure S1) and negative controls (data not shown) stained negative.

In vitro differentiation towards undesired lineages (i.e. myogenic, endothelial, or smooth muscle differentiation) results in an increase of the expression of typical rejection proteins, major histocompatibility complex (MHC) classes I and II [30]. It is thus of relevance that our differentiation protocol showed no increase in these

Table 3. Clinical evolution scores with average and standard deviation (STD) at different time points for the different treatment groups: 1 = platelet-rich plasma (PRP), 2 = mesenchymal stem cells (MSCs), 3 = PRP+MSCs, and 4 = PRP+chondrogenic induced MSCs.

		6 weeks	12 weeks	6 months	12 months
GROUP 1	Horse 1	4	2	2	1
	Horse 2	4	5	4	4
	Horse 3	3	3	3	3
	Horse 4	3	2	2	2
	Horse 5	3	3	3	3
	Average	**3.4**	**3**	**2.8**	**2.6**
	STD	0.5	1.2	0.8	1.1
GROUP 2	Horse 6	3	4	5	5
	Horse 7	0	1	2	2
	Horse 8	3	4	5	5
	Horse 9	4	4	5	4
	Horse 10	5	5	5	5
	Average	**3**	**3.6**	**4.4**	**4.2**
	STD	1.9	1.5	1.3	1.3
GROUP 3	Horse 11	3	4	4	5
	Horse 12	4	5	5	3
	Horse 13	4	5	5	4
	Horse 14	5	5	5	5
	Horse 15	3	4	4	4
	Average	**3.8**	**4.6**	**4.6**	**4.2**
	STD	0.8	0.5	0.5	0.8
GROUP 4	Horse 16	4	4	5	5
	Horse 17	5	5	5	5
	Horse 18	4	4	4	4
	Horse 19	5	5	5	5
	Horse 20	4	5	5	5
	Average	**4.4**	**4.6**	**4.8**	**4.8**
	STD	0.5	0.5	0.4	0.4

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markers. While MHC class II expression was completely absent in both native and chondrogenic-induced MSCs, MHC class I was expressed in both types of MSCs, but at very low levels (Figure 5A & B). The positive control cells (peripheral blood mononuclear cells) on the other hand, were clearly positive for both MHC markers and confirmed antibody cross-reactivity (data not shown).

Table 5. Clinical evolution scores at 6 weeks after treatment of 15 horses with native mesenchymal stem cells (MSCs) and PRP (Combination 1) or chondrogenic induced MSCs and PRP (Combination 2).

	Combination 1	Combination 2
Horse 1	3	3
Horse 2	5	4
Horse 3	4	4
Horse 4	4	4
Horse 5	3	3
Horse 6	5	4
Horse 7	5	4
Horse 8	3	5
Horse 9	5	4
Horse 10	3	5
Horse 11	3	4
Horse 12	0	2
Horse 13	5	5
Horse 14	5	4
Horse 15	3	2
Average	3.7	3.8

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Scoring of the Clinical Lameness

Study 1. The grading and overall clinical severity scores with the corresponding initial severity scores can be found in the Figure S2. All the patients enrolled in this study had similar clinical scores immediately prior to the onset of treatment and for each patient the clinical evolution scores were calculated at the termination of the experiment. The scores for each treatment group were hence clinically comparable (Table 3). The platelet-rich plasma (PRP) treated group (#1) initially (6 weeks post injection) received an average score of 3.4, which is higher than for the MSC treated group (#2). By combining both PRP and MSCs (group #3) the initial average score was increased. The clinical score improved further in the chondrogenic induced MSCs and PRP combinational therapy (group #4). Subsequently, the average score of the PRP treated group (#1) decreased to 2.6 at one year after the treatment, indicating that the effect was short-lived. The initial

Table 4. Median, minimum (min) and maximum (max) of the early and late evolution score are indicated per treatment: platelet-rich plasma (PRP), native mesenchymal stem cells (MSCs), combination (Comb) 1 (native MSCs and PRP) or Comb 2 (chondrogenic induced MSCs and PRP).

Treatment	Early score median (min; max)	Late score median (min; max)
PRP	3.0 (2.5; 4.5)	3.0 (1.5; 4.0)
MSCs	3.5 (0.5; 5.0)	5.0 (2.0; 5.0)
Comb 1	4.5 (3.5; 5.0)	4.5 (4.0; 5.0)
Comb 2	4.5 (4.0; 5.0)	5.0 (4.0; 5.0)

The ''early'' score indicates the average of the clinical evolution scores at 6 weeks and 12 weeks, whereas ''late'' indicates the average of the clinical evolution scores at 6 months and 12 months after treatment.

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average score of 3.0 in group #2 was the lowest for all the treatment groups, due to one non-responder. The average score for group #2, however, increased to 4.4 at 6 months, and then decreased to 4.2 at one year after the treatment. Horses in group #3 had a higher initial average score of 3.8, analogous to the PRP treated group, that increased to an average score of 4.2 at one year post injection, likely attributed to the long-term effects of the MSCs. Noteworthy, the average score of group #4 was initially 4.4 and increased further to 4.8 from 6 months to one year post injection. Moreover, two horses in group #4 showed functional recovery as early as 6 weeks after commencing treatment and remained sound throughout the entire study. Indeed, all horses in group #4 exhibited at least a score 4 at 6 weeks after treatment; 4 out of 5 horses in group #4 were sound at one year post injection and one horse had a score of 4 throughout the entire study period. For the first study, an overview of the average evolution scores after each treatment is presented in Figure 6A. To get a stronger short-term (early) versus long-term (late) clinical evolution in response to the different therapies the scores at 6 weeks and 12 weeks and 6 months and 12 months were added together (Table 4). The combined treatments were significantly better than the PRP treatment alone, both for the early evolution score ($P = 0.033$) and the late evolution score ($P = 0.012$). No significant differences were found between the combined treatment and the MSC treatment alone. The combined use of chondrogenic-induced MSCs and PRP generated the highest evolution scores, although the difference was not significantly higher than the combined use of native MSCs and PRP for either the early ($P = 0.530$) or late evolution score ($P = 0.207$).

Study 2. For this reason, the second clinical study was performed in which a total of 30 horses were treated with either native MSCs plus PRP (Combination 1, $n = 15$) or with chondrogenic-induced MSCs plus PRP (Combination 2, $n = 15$). The horses were only evaluated at the first time point (i.e. 6 weeks post injection). Our results show that 53% (8/15) of the horses in the first group received an evolution score 4 or more, versus 73% (11/15) in the second group. However, in both treatment groups the average evolution score was approximately the same (3.7 vs 3.8) and no statistically significant ($P = 0.67$) difference could be noticed (Table 5, Figure 6B).

Discussion

The isolated cells in the present study fulfilled all the requirements to be typed as mesenchymal stem cells (MSCs) according to the proposed guidelines by Dominici in 2006 [31]. Moreover, it has been reported that frozen equine peripheral blood (PB)-derived MSCs do not lose their stem cell characteristics [32] and that fresh equine PB-derived MSCs dramatically decline in cell number after 12 hours of transport and have a higher risk of becoming senescence after 24 hours of transport [33]. Therefore, the use of frozen samples was justified in this study, added to the product shelf life and standardized the treatments.

This study reports successful *in vitro* chondrogenic induction of equine PB-derived MSCs, followed by an *in vivo* investigation in which the therapeutic potential of chondrogenic-induced MSCs plus platelet-rich plasma (PRP) for the treatment of degenerative joint disease (DJD) was compared to native MSCs and/or PRP in 20 horses and both combination therapies in 30 horses. *In vitro* analysis of chondrogenic-induced MSCs showed decreased expression of the proliferation marker Ki67, indicating terminal differentiation with reduced proliferative capacities of the MSCs. Chondrogenic differentiation was further confirmed by increased mRNA levels of aggrecan, collagen type II and cartilage oligomeric matrix protein (COMP) as well as increased synthesis of glycosaminoglycans.

Apart from these typical chondrogenic markers, we also investigated the expression of the typical "rejection" proteins, major histocompatibility complex (MHC) class I and II, as well as of p63, a member of the p53 tumor suppressor gene family. MHC expression was evaluated because it has been reported that differentiation of allogenic MSCs towards myogenic lineages induced immunogenicity (by increasing MHC levels) [30]. In the present study, we were able to demonstrate that MHC class I was expressed at low levels in native MSCs, whereas neither MHC class II nor p63 were expressed in native MSCs, in agreement with previous reports [10,34–36]. Three days of chondrogenic induction did not alter MHC levels, reflecting low immunogenicity, which would be permissive for allogenic transplantations. However, chondrogenic induced cells clearly expressed p63, which is a typical epithelial stem cell marker [37,38]. In this regard, it has been reported that p63 plays a pivotal role in embryonic skeletal development and that p63 expression in hypertrophic chondrocytes would accelerate endochondral ossification [39]. Whether the expression of this protein enhanced cartilage repair in this study remains to be shown.

In the present study, we applied PRP and MSCs either alone, or in combination (with or without chondrogenic induction). Usage of PRP was anticipated to improve the clinical outcome as it has been reported that PRP enhances MSC proliferation and chondrogenic differentiation [40]. Although no statistically significant improvement in clinical signs of fetlock joint arthrosis could be noticed after the addition of PRP to MSCs (in comparison to MSCs alone), short-term clinical evolution scores clearly improved (3 vs 3.8 at 6 weeks and 3.6 vs 4.6 at 12 weeks post injection). Moreover, both combination therapies significantly improved the early and late clinical evolution scores in comparison to PRP treatment alone. The effect presented here differs from that of a previous study in deep digital flexor tendon lesions in Bergamasca sheep, where the addition of autologous PRP to PB-derived MSCs did not enhance regeneration [41]. In rat Achilles tendon lesions on the other hand, synergistic effects of PRP and tendon stem cells were demonstrated and resulted in the increased expression of tendon-healing genes [42]. In agreement with our study, it has also been reported that short-term beneficial effects might be expected from PRP treatment, although the exact mechanism or causative agent(s) are currently unknown [43]. It should also be taken into consideration that patient to patient heterogeneity at the time of blood sampling would result in PRP samples of varied potencies, between independent donors and within a given donor, helping account for the contradictory results obtained in previous studies [44]. Clearly, more research is warranted to determine the stimulatory or inhibitory factors present in PRP samples.

As we utilized only one batch of allogenic PRP and MSCs in the present study (i.e. PRP and MSCs from one donor), the experimental paradigm was more standardized for all patients, allowing a more accurate comparison between the different treatment groups. In this regard, Carrade et al. have reported that a single intra-articular injection of allogenic MSCs in healthy equine joints induced a similar immune response as an autologous injection [22]. Moreover, in a study by Guest et al., no cell-mediated immune response was detected at all after allogenic MSC injection in equine superficial digital flexor tendon lesions [45]. Analogously, in this study, there were no indications of an immune response after allogenic MSC or PRP treatment. Nonetheless, no definite conclusion can yet be made concerning the immunogenicity of both allogenic therapies used in this study.

The major advancement of this study is the application of MSCs in equine patients with naturally occurring fetlock joint arthrosis, rather than in horses with experimentally induced cartilage lesions. The fact that experimentally induced cartilage lesions may only partially resemble the naturally occurring arthrosis may explain why previous studies [15–17] were not able to detect clinical improvement, in contrast to our study. However, while clinical improvement was absent in the aforementioned studies, an early beneficial impact on histologic appearance and biochemical composition [17] as well as a late enhancement of aggrecan levels [16] was observed. Frisbie et al. [15] reported no significant clinical or histological effect within 70 days after treatment with bone marrow-derived MSCs in the middle carpal joint of horses, but did observe improvements in synovial fluid PgE2 levels, which would ultimately inhibit the production of pro-inflammatory cytokines [46].

In contrast with previous equine reports, and in agreement with the present study, it has been described that carpal joint arthrosis in donkeys improved clinically and radiographically at 2 months and 6 months after treatment with bone marrow-derived MSCs [47]. Moreover, green fluorescent protein-labelled MSCs integrated in the cartilage, which indicated that the MSCs participated in the healing process of the damaged tissue. Whether the difference in location and structural composition of the joints, the experimental model, the MSC samples, or even the carrier used in the aforementioned studies were responsible for the lack of clinical improvement remains to be proven.

While our study provides evidence of clinical improvement with MSC therapy in the fetlock joint, it must be noted that the initial clinical severity scores were mild to moderate (4–5 on 10), indicating that these patients were not in the last stage of osteoarthritis. Clearly, the mechanisms underlying this effect are unclear and will need to be investigated in the future. Furthermore, patients in earlier and later stages of osteoarthritis and larger sample numbers under double-blinded evaluation criteria will eventually need to undergo a similar procedure as described here to improve statistical power and allow for more definite conclusions. In our first preliminary study reported here, only five horses per treatment were evaluated, which could have been the reason why no statistical significant difference was observed between the evolution scores of both combination therapies (i.e. PRP+native MSCs versus PRP+induced MSCs). Although in a larger group of patients substantially more horses received a score of 4 or more in the second combination therapy, the average evolution scores of both combination therapies were not significantly different. Indeed, further optimization is necessary to ultimately assess both therapies.

In conclusion, our results indicate that chondrogenic induction can be achieved in equine MSCs and that the combined use of PRP and MSCs (chondrogenic induced or not) significantly improved the functionality and sustainability of damaged joints in horses with mild to moderate lameness, due to fetlock joint osteoarthritis, up to 12 months post treatment. The highest clinical scores were noticed upon treatment with the chondrogenic induced MSCs and PRP. Nonetheless, more protracted studies need to be performed to confirm the positive effects of chondrogenic induction.

Supporting Information

Figure S1 Immunocytochemistry on cytospins using Ki67 (A), collagen (Col) type II (B), vimentin (C) and p63 (D). Native mesenchymal stem cells (MSCs) were negative for p63 and positive for Ki67, Col II and vimentin, whereas chondrogenic induced MSCs were positive for p63, Col II and vimentin and slightly positive for Ki67. Arrows indicate a decreased signal for Ki67 in some chondrogenic induced MSCs. The relevant isotype controls were negative. Scale bar represents 25 μm.
(TIF)

Figure S2 Clinical grading and severity scores for the different treatment (PRP = platelet-rich plasma, MSC = mesenchymal stem cell, IND = chondrogenic induced mesenchymal stem cell) groups at different time points. The sum of the clinical grading according to the American Association of Equine Practitioners (AAEP) on 5, the flexion test on 3 and joint effusion evaluation on 2 gave an overall clinical severity score on 10.
(TIF)

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Author Contributions

Conceived and designed the experiments: SB MZ SC MS TM SJF KC LD AFO KW JHS. Performed the experiments: SB MZ SC MS TM SJF KC LD AFO KW JHS. Analyzed the data: SB MZ SC MS TM SJF KC LD AFO KW JHS. Contributed reagents/materials/analysis tools: KC JHS KW SJF. Wrote the paper: SB JHS KW.

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ADDITIONAL READING

Culture and characterisation of equine peripheral blood mesenchymal stromal cells.

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Chondrogenic priming at reduced cell density enhances cartilage adhesion of equine allogeneic MSCs - a loading sensitive phenomenon in an organ culture study with 180 explants.

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NOTES

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