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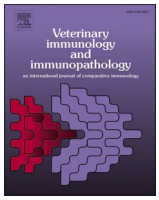

Research paper

Repeated intra-articular administration of equine allogeneic peripheral blood-derived mesenchymal stem cells does not induce a cellular and humoral immune response in horses

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ABSTRACT

Objective: The use of mesenchymal stem cells (MSCs) for the treatment of equine joint disease is widely investigated because of their regenerative and immunomodulatory potential. Allogeneic MSCs provide a promising alternative to autologous MSCs, since the former are immediately available and enable a thorough donor screening. However, questions have been raised concerning the immunogenic potential of allogeneic MSCs, especially after repeated administration.

Methods: Current retrospective study assessed the cellular and humoral immunogenicity of ten jumping and dressage horses with naturally occurring degenerative joint disease which were treated 3 times intra-articularly with a 1 mL stem cell suspension containing 1.4–2.5 million chondrogenic induced equine allogeneic peripheral blood-derived MSCs (ciMSCs) combined with 1 mL equine allogeneic plasma. Stem cells from 2 donor horses were used. Horses were clinically evaluated for joint effusion, presence of pain to palpation and skin surface temperature at the local injection site, joint range of motion, occurrence of adverse events and the presence of ectopic tissue. The cellular immune response was analyzed using a modified mixed lymphocyte reaction and the humoral immune response was investigated using a flow cytometric crossmatch assay by which the presence of alloantibodies against the ciMSCs was evaluated. Presence of anti-bovine serum albumin antibodies was detected *via* ELISA.

Results: Clinical evaluation of the horses revealed no serious adverse effects or suspected adverse drug reactions and no ectopic tissue formation at the local injection site or in other areas of the body. Generally, repeated administration led to a decrease of horses with joint effusion of the affected joint. Pain to palpation, skin surface temperature and joint range of motion did not increase or even decreased after treatment administration.

Abbreviations: 7-AAD, 7-aminoactinomycine-D; AB/AM, antibiotics/antimycotics; AT, adipose tissue; BM, bone marrow; BSA, bovine serum albumin; CD, cluster of differentiation; CFSE, carboxyfluorescein succinimidyl ester; ConA, concanavalin A; ciMSC(s), chondrogenic induced equine allogeneic peripheral blood-derived mesenchymal stem cell(s); EDTA, ethylenediaminetetraacetic acid; FBS, foetal bovine serum; HBSS, hank's balanced salt solution; PB, peripheral blood; PBMCs, peripheral blood mononuclear cells; MHC, major histocompatibility complex; MLR, mixed lymphocyte reaction; MSC(s), mesenchymal stem cell(s).

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Allogeneic ciMSCs did not induce a cellular immune response and no alloantibodies were detected in the recipients' serum, regardless the presence of BSA antibodies in 70 % of the horses.
Conclusion: Repeated intra-articular injections with allogeneic equine ciMSCs did not elicit clinically relevant adverse events. Furthermore, current study indicates the absence of a cellular or a humoral immune response following repeated intra-articular injections.

1. Introduction

Just like in human medicine, inflammatory and degenerative joint disease is a common affection in equine practice. Approximately 30 % of horses suffer from inflammatory joint disease or from chronic degenerative joint disease, making these a major cause of diminished athletic function and early retirement in equine performers (Neundorf et al., 2010; de Souza, 2016a).

Currently, available treatments only decrease clinical symptoms or enhance recovery for a short period of time. The currently most commonly used medicinal treatments are nonsteroidal and steroidal anti-inflammatory drugs and disease-modifying drugs (*e.g.* hyaluronic acid, chondroitin sulfate or glucosamine). However, none of these conventional therapies stops the progression of the disease, resulting in no durable solutions for degenerative joint disease (Bogers, 2018; Contino, 2018; Johnson and Frisbie, 2016; de Souza, 2016b).

Recently, promising advances in the therapy of joint disease were obtained in the field of regenerative medicine. According to *in vitro* observations, cell-based therapies are a valuable alternative because they have the potential to produce cartilage specific substances, such as collagen type II and aggrecan (Berg et al., 2009; Spaas et al., 2015). Furthermore, long-term clinical improvement and pain relief in affected joints of both horses and humans were achieved with intra-articular injection of mesenchymal stem cells (MSCs) (Broeckx et al., 2014a; Ferris et al., 2014; Spasovski et al., 2018). The intra-articular use of autologous and allogeneic MSCs has even shown promising results for the enhancement of cartilage repair (Berg et al., 2009; Schnabel et al., 2013; Broeckx et al., 2014a, b). These studies report the safety and efficacy of stem cell treatments in inflammatory joint disease, based on the number of horses that were able to return to work or to previous levels of performance (Broeckx et al., 2014a; Ferris et al., 2014). However, it is still unclear whether allogeneic peripheral blood derived MSCs elicit an immune response in horses, especially when administered repeatedly. In recent studies it was reported that allogeneic MSCs are capable of inducing an immune response *in vitro* and *in vivo* (Schnabel et al., 2014; Berglund and Schnabel, 2017). Regarding the safety of repeated administration of allogeneic MSCs *in vivo,* the literature reports are controversial (Ardanaz et al., 2016; Barrachina et al., 2020; Joswig et al., 2017). According to Ardanaz et al. (2016) allogeneic bone marrow-derived MSCs (BM-MSCs) can be safely used when repeatedly administered intra-articularly. In contrast, Joswig et al. (2017) reported an adverse response (*i.e.* increased cellular infiltrate in synovial joints injected with allogeneic MSCs) following repeated injections with BM-MSCs. In the past, multiple researchers have administered allogeneic MSCs repeatedly, trusting reports and clinical observations without any major side effects (Horwitz et al., 2002; Ardanaz et al., 2016; Beerts et al., 2017; Joswig et al., 2017). In contrast to autologous MSCs, allogeneic MSCs provide the benefit of ready to use, off the shelf availability and high quality selected donor horses. The peripheral blood provides as a minimally invasive MSC source, so harvesting can be performed repeatedly with minimal donor sites morbidities and little discomfort for the animal. Moreover, the environment in a degenerated joint might not provide the correct stimulus for MSC differentiation and signaling or alternatively, may even negatively influence their functionality. Therefore, chondrogenic induction of MSCs may improve the clinical outcome (Broeckx et al., 2019a, b). Until now, comprehensive studies investigating the immunogenicity of equine allogeneic peripheral blood derived MSCs after repeated administration in horses are lacking, despite being of great importance for further development of MSC based treatments of degenerative joint disease. Owens et al. investigated the immunological aspects following repeated injections of allogeneic adipose tissue- or BM-MSCs in horses by assessing alloantibody development (Owens et al., 2016). In the latter study, alloantibody development was reported after horses with different pathologies (*i.e.* tendon lesions, eye pathologies) had been injected with 25–50 million MSCs per treatment administration through different routes.

The goal of this study was to investigate the clinical safety and the cellular and humoral immunogenicity of equine allogeneic chondrogenic induced peripheral blood derived MSCs after repeated intra-articular administration. Therefore, a retrospective study was set-up analyzing the blood for an immune response of 10 horses repeatedly treated with chondrogenic induced peripheral blood derived MSCs.

2. Materials and methods

2.1. Study design

Clinical records of 10 jumping and dressage horses with naturally occurring degenerative joint disease treated at least 3 times (minimal treatment interval of 3 weeks) intra-articularly with 1 mL stem cell suspension containing 1.4–2.5 million chondrogenic induced equine allogeneic peripheral blood-derived MSCs (ciMSCs) combined with 1 mL equine allogeneic plasma were evaluated in this retrospective study. ciMSCs and EAP were thawed and administered as previously described (Broeckx et al., 2019a). Stem cells originated from 2 donor horses. At the time of each intra-articular equine ciMSC administration, all horses received a single oral dose of non-steroidal anti-inflammatory drugs (Meloxicam, 15 mg/mL) before treatment. To assess the cellular and the humoral immune response, peripheral blood was collected on a single occasion for the assays described below, in order to reduce inter-test variation. This means that the days after the last treatment and collection of blood varied for each of the 10 horses as follows: 5-19-53-57-74-102-150-164-270-553 days after the last treatment. An informed consent for blood sampling was obtained from each horse owner. Blood sampling was approved by the Committee on the Ethics of Animal Experiments (EC_2016_003) of Global Stem cell Technology (permit Number: LA1700607).

2.2. Horses

The 10 recipient warmblood horses consisted of 4 mares and 6 stallions. The age at the time of the blood collection ranged between 7 and 17 years. The affected joints consisted of distal interphalangeal joints, the proximal interphalangeal joints, the metacarpophalangeal joints and femorotibial joints. The disease stage varied between mild joint inflammation and the presence of small osteophytes to severe osteoarthrosis with deformity of the bone contour, severe sclerosis and narrowing of the joint space. The location of the lameness was confirmed with intra-articular anesthesia. Time between injections varied between 21 and 238 days. The blood collection was performed at 5–553 days following the last treatment with ciMSCs The two ciMSC donors are warmblood horses (1 mare and 1 gelding with an age of 9 and 10 years). The Aa^{+} red blood cells and the blanco serum needed for the flow cytometric crossmatch assay (see below) are derived from two warmblood horses (a 10 year old gelding and a 23 year old gelding respectively).

2.3. Isolation and chondrogenic induction of mesenchymal stem cells

Chondrogenic induced MSCs were prepared from peripheral blood (PB) of two different adult donor horses according to good manufacturing practice (GMP certificate no. BE/GMP/2015/082) and characterized as previously described by the same group (Broeckx et al., 2012). Blood collection of the donor horses was approved by the local ethical committee (approval number: EC_2012_001 and EC_2016_003) and the horses were screened for transmittable diseases by different clinical laboratories in agreement with the Belgian Federal and European Medicines Agency. Briefly, 50 mL of blood was collected in sterile ethylenediaminetetraacetic acid (EDTA) tubes from the *vena jugularis* from both donor horses. The blood was centrifuged and the buffy coat was collected, diluted and layered upon an equal amount of Percoll (GE Healthcare) for gradient centrifugation. After washing, isolated cells were cultured in expansion medium consisting of Dulbecco's modified Eagle's medium (Life Technologies) supplemented with fetal bovine serum (FBS, Sigma-Aldrich) and a combination of antibiotics and antimycotics (AB/AM, Sigma-Aldrich). At passage 5, cells were characterized by evaluation of the presence (Cluster of Differentiation (CD) 29, CD44 and CD90) and absence (Major Histocompatibility Complex (MHC) II and CD45) of specific cell surface markers using by flow cytometry as previously described (Spaas et al., 2013). Consequently, the cells were seeded in two dimensional tissue culture systems for chondrogenic induction using a medium supplemented with FBS and growth factors as previously described by the same group (Spaas et al., 2015). At the next confluency, ciMSCs were trypsinized, resuspended in Dulbecco's modified Eagle's medium supplemented with 10 % dimethyl sulfoxide (Sigma-Aldrich) and 1.4–2.5 million ciMSCs per vial were frozen until further use (−80 °C).

2.4. Clinical evaluation

All horses underwent a complete clinical evaluation before and 6 ± 2 weeks after each injection. If the next treatment was within this period, a complete clinical evaluation was performed prior to injection. This included a general clinical examination to evaluate the presence of adverse events and ectopic tissue at the injection site or in other areas of the body, determination of joint effusion, assessment of the presence of pain to palpation and increased skin surface temperature at the injection site, and an evaluation of the presence of an abnormal range of motion of the treated joint (Table 1). Additionally, horses were re-examined 24 and 48 h (±6 h) after the first and second injection and a follow-up examination was scheduled 6 ± 2 weeks after the third ciMSC administration using the parameters as previously described (joint effusion, skin surface temperature and presence of pain to palpation at the injection site and joint range of motion). The animal caretaker was requested to evaluate and report increased pain to palpation, joint effusion and skin surface temperature at the injection site the first and second day after the third injection.

Table 1
Clinical scores used in all patients to assess joint effusion, skin surface temperature and pain to palpation at the injection site and range of motion of the injected joint.

Parameter	Score system	Definition
Joint effusion	0	No joint effusion
	1	Mild joint effusion
	2	Moderate joint effusion
	3	Severe joint effusion
	4	Extreme (peri-articular) joint effusion
Skin surface temperature at the injection site	No	No increased skin surface temperature noted
	Yes	Increased skin surface temperature noted
Pain to palpation at the injection site	No	No pain to pressure noted
	Yes	Pain to pressure noted
Range of motion of the injected joint	Normal	Normal range of motion noted
	Abnormal	Decreased range of motion noted

3. Mixed lymphocyte reaction (MLR)

3.1. Blood collection and preparation of peripheral blood mononuclear cells

Peripheral blood mononuclear cells (PBMCs) were successfully isolated from whole blood 5–553 days after the last treatment administration and further cultured according to internal specifications from six out of ten horses. For three horses, the isolated PMBCs could not be used due to red blood cell contamination in the interphase (probably due to small blood clots present in the original EDTA tubes). Additionally, the PBMCs of one horse could not to be stimulated by the used mitogen (see below).

From each horse, 18 mL of whole blood was collected in sterile EDTA tubes and kept overnight at 4 °C. Subsequently, the blood was centrifuged and the buffy coat was collected and diluted in Hank's balanced salt solution (HBSS, Life Technologies). Next, the suspension was layered upon an equal volume of Percoll and the interphase was collected after gradient centrifugation. PBMCs were washed and resuspended in HBSS to a final concentration of 1×10^6 PBMCs/mL.

To be able to evaluate proliferation, PBMCs were labelled with carboxyfluorescein succinimidyl ester by manufacturing work instructions (CFSE, Life Technologies). Briefly, CFSE staining solution was added to the PBMC cell suspension (1×10^6 PBMCs/mL) in a 1:1000 dilution. After 20 min of incubation, expansion medium, as described above in culture process, was added to block the staining process. After centrifugation, the PBMCs were washed and resuspended in fresh expansion medium supplemented 2-mercaptoethanol (Sigma-Aldrich) to a final concentration of 2.5×10^6 PBMCs/mL.

3.2. Modified MLR for immunogenicity

To investigate the ability of ciMSCs to induce a cellular immune response, a modified one-way MLR was performed in a U-bottom 96-well tissue-culture plate. Isolated PBMCs were used immediately without cryopreservation. All samples and controls were seeded in duplicate. The negative control consisted of a PBMC culture alone (to assess baseline PBMC proliferation). PBMCs stimulated by the mitogen concanavalin A (ConA, 5 μg/mL, Sigma-Aldrich) were included as a positive control.

For the samples, ciMSCs were thawed, washed and resuspended in cell culture medium to a final concentration of 2.5×10^5 ciMSCs/mL. The ciMSCs were plated at a ratio of 1:10 ciMSC:PBMCs. The PBMCs from each repeatedly treated horse were co-incubated with ciMSCs from the same donor from which they had received treatment (ciMSCs treated donor) and with ciMSCs from the other donor included in the study which was an unrelated donor (ciMSCs unrelated donor). Cultures were maintained for 4 days in 5% CO_2 at 37 °C with medium supplemented with FBS, AB/AM and 2-mercaptoethanol. Over these 4 days, the media were not refreshed.

3.3. Immunomodulatory assay

Five horses had a PBMC yield being high enough to perform also a modified MLR for the assessment of immunomodulation. PBMCs were labelled with CFSE and seeded in a U-bottom 96-well plate in duplicate as described above. The negative and positive controls consisted of respectively PBMCs alone and ConA stimulated PBMCs. In order to investigate the immunomodulatory properties of ciMSCs, PBMCs were treated with ConA as described earlier prior to incubation with ciMSCs. Again, ciMSCs were added at a ratio of 1:10 ciMSC:PBMCs, by which

PBMCs from each horse were co-incubated with ciMSCs from the same donor from which they received treatment (ciMSCs treated donor) and with ciMSCs from an unrelated donor (ciMSCs unrelated donor). Cultures were maintained for 4 days in 5 % CO_2 at 37 °C without exchange of media over this culture period.

3.4. Flow cytometry analysis

After the 4 days incubation period, PBMC proliferation was evaluated using flow cytometry. All samples including the negative and positive control samples were pelleted and stained with 7-aminoactinomycine D (1:100, 7-AAD, BioLegend) to visualize cell viability. Next, T-lymphocytes and plasma cells in the PBMC population were detected using antibodies against CD3 and CD138 respectively. Samples for CD3 staining were pelleted and a primary unconjugated mouse anti-horse CD3 antibody (1:5; clone UC F6G-3.3; Laboratory of Dr. J. Scott, University of California Davis, Davis, CA, USA) was added for 30 min. Next, these samples were washed, pelleted and stained with a secondary AlexaFluor 647 donkey anti-mouse antibody (1:100; Jackson Immunoresearch) for 20 min in the dark. The optimal dilution of the secondary AlexaFluor 647 donkey anti-mouse antibody (Jackson Immunoresearch) was determined through a preliminary titration experiment (tested 1:25 up to 1:150 dilution of the stock concentration) during which it was also investigated if a blocking step with donkey serum was necessary to prevent the aspecific binding of secondary antibody. After the incubation period, the cells were stained with 7-AAD. The samples for CD138 staining were centrifuged and incubated with a PE/Cy7 conjugated anti-human CD138 antibody (1:10; clone; DL-101; BioLegend) for 15 min in the dark at 4 °C. Cross-reactivity of the equine PBMCs with this CD138 was shown in a preliminary titration experiment (data not shown). After this incubation period, the cells were also stained with 7-AAD. In order to include only single, viable cells in the analysis and eliminate any dead cells, doublets or debris, the PBMC population was gated using forward and side scatter and double doublet discrimination was performed. Viable PBMCs were gated on the 7-AAD staining. Within this viable population, the proliferation of the PBMCs was measured using CFSE staining. CD3 and CD138 positive cells were determined in the proliferated PBMC proliferation. Flow cytometry analysis was performed on the BD FACSCanto II.

4. Flow cytometric crossmatch assay

4.1. Blood collection and preparation of red blood cells and equine sera

For the positive control, 6 mL of whole blood from an Aa^+ horse was collected in EDTA blood collection tubes. After centrifugation to remove plasma and the buffy coat, the red blood cells (RBCs) were diluted in HBSS to a final concentration of 10^6 RBCs/mL.

For the negative control, 9 mL of equine whole blood was collected in serum clot activator blood collection tubes from a horse that had never received a MSC injection before. After coagulation, this equine serum (*i.e.* blanco serum) was collected following centrifugation. Next, the blanco serum was heat-inactivated at 56 °C for 30 min.

For the samples, 9 mL of whole blood of each horse was collected in serum clot activator blood collection tubes and allowed to clot for 2 h at room temperature. After centrifugation, the sera were collected and heat-inactivated at 56 °C for 30 min.

4.2. Flow cytometric crossmatch assay

The flow cytometric crossmatch assay was performed according to Owens et al. (2016). Briefly, RBCs derived from an Aa^+ horse were incubated with serum derived from an Aa^- horse (Aa^- antiserum; UC Davis, USA). Following incubation, 100 μL of the secondary antibody (1:100; AlexaFluor 647 Goat anti-Horse IgG, Jackson Immunoresearch) was added to the cell pellet and incubated for 20 min at room temperature in the dark. Next, the RBCs were washed two times and finally resuspended in flow stain buffer for flow cytometric analysis on the BD FACSCanto II. This sample was included as a positive control to verify correct functioning of the secondary antibody.

The vial containing ciMSCs was thawed in the palm of a hand and the cells were resuspended in HBSS to a final concentration of 2×10^5 MSCs/mL. One mL of the MSC cell suspension was centrifuged and then blocked with 5 % normal goat serum.

As a first negative control, ciMSCs were incubated with the secondary antibody (1:100) for 20 min at room temperature without adding equine serum. After incubation, the sample was centrifuged, resuspended in flow stain buffer and stained with 7-AAD (1:100, BioLegend) for flow cytometric analysis.

As a second negative control, ciMSCs were incubated with the heat-inactivated blanco serum (see above) for 30 min at room temperature. Next, 100 μL of the secondary antibody (1:100; AlexaFluor 647 Goat anti-Horse IgG, Jackson Immunoresearch) was added to the cell pellet and incubated for 20 min at room temperature in the dark. The sample was centrifuged, resuspended in flow stain buffer and stained with 7-AAD (1:100, BioLegend) for flow cytometric analysis. This sample was used to determine background binding of equine serum to the ciMSCs.

For the recipient serum samples, the ciMSCs were incubated with the heat-inactivated recipient serum for 30 min. Following centrifugation, a secondary antibody (1:100) staining of the ciMSCs was performed as described earlier. After incubation all samples were centrifuged and stained with 7-AAD for flow cytometry analysis. All samples were measured on the BD FACSCanto II (Software: FACS Diva).

4.3. Detection of anti-BSA antibodies

An ELISA was adapted from Gershwin et al. (2012) and Owens et al. (2016) to detect antibodies directed against bovine serum albumin (BSA). In short, a 96-well ELISA plate (PerkinElmer) was coated with 100 μL BSA (1 μg/well; Sigma, St. Louis, MO) in a carbonate-bicarbonate buffer (63.5 mM carbonate, pH 9.6) overnight at 4 °C. Next, 100 μL of 1 % rabbit serum albumin (Sigma, St. Louis, MO) diluted in HBSS was added to each well and incubated for 1 h at 37 °C. Wells were washed using HBSS + 0.1 % Tween 20 for 10 min, followed by 6 brief wash steps. Subsequently, 100 μL of the test serum (diluted 1:100 in wash buffer) was added to each well. Each sample was plated in duplicate. Negative controls consisted of phosphate buffered saline and fetal horse serum. Positive samples were obtained from horses with known anti-BSA antibody binding. The plate was incubated for 1 h at 37 °C and washed as described above. Next, 100 μL of rabbit anti-equine IgG H&L-HRP (diluted 1:100,000; Abcam) was added to each well. The plate was incubated at 37 °C for 1 h and washed as described above. 100 μL of TMB Peroxidase Substrate (KPL, Gaithersburg, MD) was added to each well and incubated at room temperature in the dark. The colorimetric reaction was stopped by adding 100 μL of 2 N H_2SO_4 to each well. Finally, the plate was read at 450 nm on a microplate reader (Victor PerkinElmer). The fold increase in color relative to the negative control was determined for each sample.

5. Data analysis

5.1. Clinical evaluation

For the clinical evaluation, data was imported in spreadsheet software and analyzed descriptively.

5.2. Mixed lymphocyte reaction

The data were analyzed using the fold change of each coculture/positive control as compared to the negative control. Therefore, the negative control was set to 1 and a mixed model was fitted. The data were analysed in two different ways. First, the fold changes were

classified per ciMSC donor horse, independent of whether the recipient received cells derived from this donor horse during treatment. Secondly, the data were rearranged so that the fold changes were classified per ciMSC donor horse as received during treatment. The Bonferroni multiple comparisons adjustment technique was applied setting the comparison wise significance level for 4 comparisons to $0.05/4 = 0.0125$. Equivalence of treatments was investigated using 95 % confidence intervals.

5.3. Flow cytometric crossmatch assay

Data were analyzed in IBM SPSS Statistics for Windows, version 25 software for statistical analysis. Since data were not normally distributed, an independent samples Mann-Whitney *U* test was used for analysis. P-values below 0.05 were considered statistically significant.

6. Results

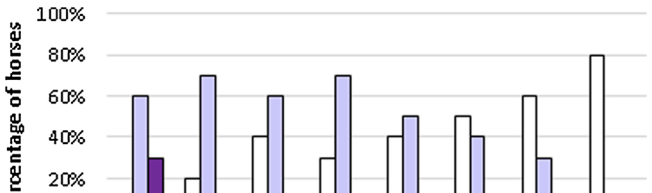
6.1. Isolation and characterisation of ciMSCs

The cells displayed all properties to be characterized as MSCs before the chondrogenic induction. Briefly, they were plastic-adherent, trilineage differentiation towards osteoblasts, chondroblasts and adipocytes was successful and MSCs were positive for CD29 (100 %), CD44 (100 %), CD90 (100 %) and negative for CD45 (1 %) and MHC II (0 %). The average population doubling time over 10 passages was 1.4 and 2.5 for donor horse 1 and 2, respectively and passage 10 ciMSCs displayed 95 % viability, a MSC immunophenotype (100 % CD44, 100 % CD90, and 0 % MHC II) and 6.0 and 17.3 fold (donor horse 1 and 2 respectively) cartilage oligomeric matrix protein increase as a marker for chondrogenic induction (Broeckx et al., 2014b).

6.2. Clinical evaluation

No serious adverse events or suspected adverse drug reactions were observed and no ectopic tissue was found at the injection site or in other areas of the body during the clinical evaluations.

Before treatment (T1) 10 % of the horses showed no joint effusion and 90 % of the horses showed a mild (60 % score 1) to moderate (30 % score 2) effusion of the affected joint (Fig. 1). One day (T1.1) and two days (T1.2) after the first treatment, horses without joint effusion increased to 20 % and 40 %, respectively. Also one day after the first injection (T1.1), 70 % of the horses showed a mild joint effusion which decreased to 60 % on the second day after treatment (T1.2).



	T1	T1.1	T1.2	T2	T2.1	T2.2	T3	T3.1
Score 0	10%	20%	40%	30%	40%	50%	60%	80%
Score 1	60%	70%	60%	70%	50%	40%	30%	10%
Score 2	30%	10%	0%	0%	10%	10%	10%	10%

Fig. 1. Effusion score before and 6 ± 2 weeks after each treatment. Score 0: no effusion, score 1: mild effusion and score 2: moderate effusion. T1: before treatment and day of the first treatment. T1.1: one day after the first treatment. T1.2: two days after the first treatment. T2: day of the second treatment. T2.1: one day after the second treatment. T2.2: two days after the second treatment. T3: day of the third treatment. T3.1: follow-up (6 ± 2 weeks) after the third treatment.

On the day of the second ciMSC administration (T2), 30 % of the horses had no effusion and 70 % of the horses revealed a mild effusion of the affected joint. One day (T2.1) and two days (T2.2) after treatment, respectively 40 % and 50 % of the horses showed no joint effusion. Horses with a mild joint effusion decreased to 50 % (T2.1) and 40 % (T2.2), but one horse showed a moderate joint effusion after the second injection (T2.1 and T2.2).

On the day of the third ciMSC administration, 60 % of the horses showed no joint effusion and 40 % had a mild (30 % score 1) to moderate (10 % score 2) effusion. Finally, at the follow-up appointment (6 ± 2 weeks after the last treatment), 80 % of the horses had no effusion and 20 % showed a mild (10 % score 1) to moderate (10 % score 2) effusion of the affected joint. In conclusion, in three horses a joint effusion score of 2 was observed prior to treatment. This effusion improved in two horses within 2 days following treatment. In one horse the effusion score remained 2 for the main part of the study.

Pain to palpation at the injection site was present in 30 % of the horses before treatment. After the first treatment, none of the horses displayed pain to palpation. This was also the case after the second and third treatment.

Additionally, 20 % of the horses had an increased skin surface temperature at the injection site before treatment. After the first, second and third treatment however, only 10 % of the horses had an increased skin surface temperature.

Before treatment, 20 % of the horses displayed limited range of motion of the affected joint. This number remained the same after the first treatment but from the second day after the second treatment onwards (T2.2, T3 and T3.1), a normal range of motion of the affected joint was observed in all horses.

Finally, no increase in pain to palpation, joint effusion and skin surface temperature at the injection site was reported by the animal caretaker at 24 h and 48 h after the third injection.

6.3. Optimisation of the CD3 staining

A titration series of the secondary AlexaFluor 647 donkey anti-mouse antibody was performed with dilutions ranging from 1:25 to 1:150. The antibody concentration at the start of the plateau phase (*i.e.* 15 μg/mL) was used as a final working concentration. This corresponded to a 1:100 dilution of the stock concentration of the secondary antibody (Fig. 2 and Supplementary Fig. 1).

It was also noted that only a high concentration of secondary antibody (1:25 dilution) led to an aspecific binding when no blocking step was included (Fig. 3 and Supplementary Fig. 2). Since 1:100 was determined to be the optimal working dilution of the secondary antibody, a blocking step was not necessary and could thus be excluded.

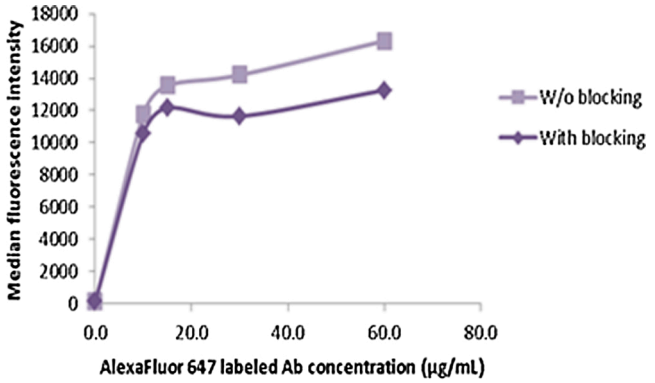


Fig. 2. Titration of the AlexaFluor 647 donkey anti-mouse antibody. The saturation curve for PBMCs stained with a primary CD3 antibody and increasing concentrations of secondary antibody with or without preliminary blocking step of the PBMCs with donkey serum.

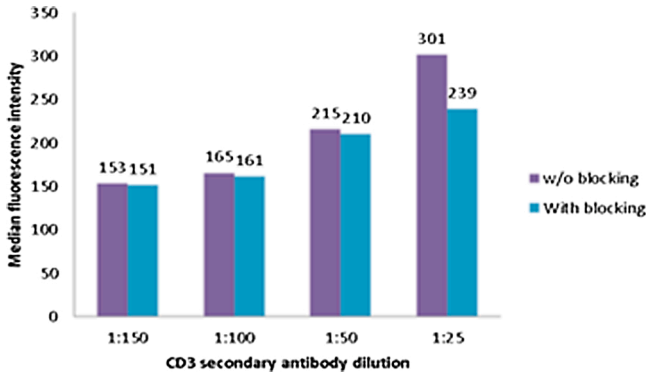


Fig. 3. Median fluorescence intensity of the secondary antibody. Comparison of the median fluorescence intensity of the secondary antibody with or without a blocking step.

6.4. Modified one-way MLR for immunogenicity

For all coculture groups, the addition of ciMSCs to PBMCs isolated from repeatedly treated horses did not lead to an increase in mean proliferation percentage compared to the negative control group (Fig. 4). The mean proliferation percentage from the PBMCs in the negative control group (3.7 ± 2 %) did thus not differ significantly from the mean proliferation percentage of the PBMCs incubated with ciMSCs from donor horse 1 (4.6 ± 0.6 %, P = 0.0452) and donor horse 2 (4.8 ± 1.2 %, P = 0.0578) (Fig. 4A). There was also no significant difference between the mean proliferation percentage of the PBMCs in the negative control group (3.7 ± 2 %) and in the coculture group with ciMSCs from the treated donor (5.1 ± 1 %, P = 0.0243) and unrelated donor (4.3 ± 0.7 %, P = 0.1031) (Fig. 4B).

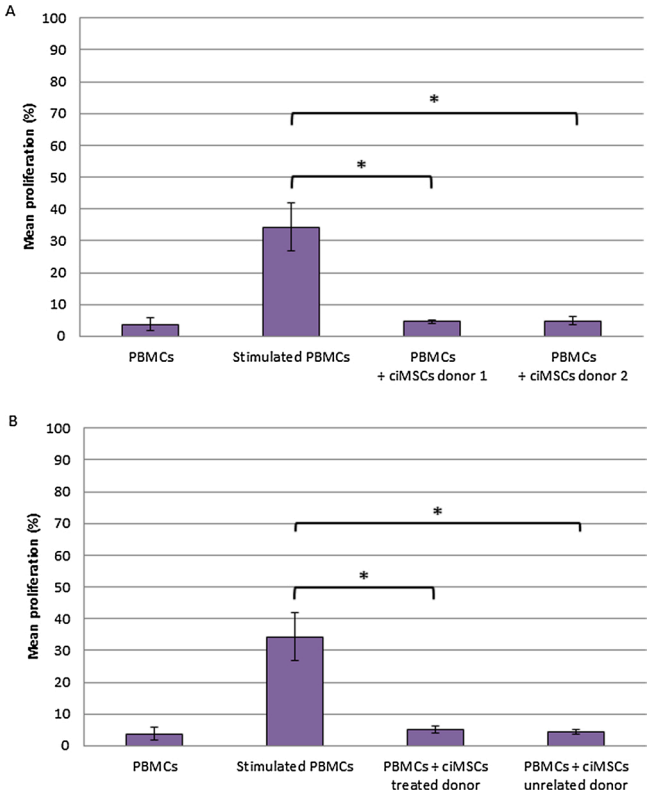


Fig. 4. Mixed lymphocyte reaction assay; immunogenicity of allogeneic ciMSCs. A. Allogeneic ciMSCs from two different donor horses cocultured with PBMCs derived from horses having received at least 3 ciMSC treatments derived from donor 1 or donor 2. **B.** Allogeneic ciMSCs derived from the treated donor or unrelated donor cocultured with PBMCs derived from horses having received at least 3 treatments of the same donor. Bars indicate the mean proliferation percentage ± SD. * P < 0.0125. PBMCs: peripheral blood mononuclear cells; ciMSCs: chondrogenic induced mesenchymal stem cells.

As no significant differences could be observed between treatment groups, 95 % confidence intervals (CI) were used to demonstrate the expected differences (Table 2).

The differences between the values captured by the 95 % confidence intervals are minor and just above 2. Therefore, the observed differences between the treatment groups are clinically irrelevant.

6.5. Immunomodulatory assay

The proliferation percentage of stimulated allogeneic PBMCs from repeatedly treated horses following coculture with ciMSCs was neither stimulated nor suppressed. No significant difference in mean PBMC proliferation could be observed between the positive control (22.5 ± 3.8 %) and the coculture samples with ciMSCs from donor horse 1 (21.1 ± 4.9 %, P = 0.4706) and donor horse 2 (22.7 ± 5 %, P = 0.96) (Fig. 5A). There was also no significant difference between the mean PBMC proliferation in the positive control group (22.5 ± 3.8 %) and in the coculture group with ciMSCs of the treated donor (22.3 ± 4.9 %, P = 0.9994) or the unrelated donor (21.4 ± 5.2 %, P = 0.6939) (Fig. 5B).

As no significant differences could be observed between treatment groups, 95 % CI were used to demonstrate the expected differences (Table 3).

The differences between the values captured by the 95 % confidence intervals are minor and just below 2. Therefore, the observed differences between the treatment groups are clinically irrelevant.

6.6. Presence of T-lymphocytes and plasma cells in the proliferation PMBC population

The presence of T-lymphocyte and plasma cell subsets was measured in the proliferative populations of ConA stimulated PBMCs (positive control group) and the ciMSC:PBMC cocultures. In the MLR for immunogenicity, no PBMC proliferation was observed when PBMCs were cocultured with ciMSCs. Therefore, the percentage of CD3 and CD138 positive cells could not be determined accurately (Supplementary Fig. 3). In the MLR for immunomodulation, both culture conditions (positive control and PBMC:ciMSC coculture) displayed a similar composition of T-lymphocytes and plasma cells, *i.e.* 7–11 % CD138+ plasma cells and 96–97 % CD3+ T-lymphocytes (Fig. 6 and Supplementary Fig. 4).

6.7. Flow cytometric crossmatch assay

No significant difference in mean percentage ciMSCs positive for alloantibody recognition was detected between the negative control group (incubation with blanco serum) (4.72 %) and the recipient serum after treatment (3.76 %). Moreover, a significant lower percentage ciMSCs positive for alloantibody recognition was detected after

Table 2
Calculations of the 95 % confidence intervals of the treatment groups as compared to the negative control.

Treatment	Estimate	SE	2*SE	95 % CI
PBMCs + ciMSCs donor 1	1.5803	0.2656	0.5312	[1.0491; 2.1115]
PBMCs + ciMSCs donor 2	1.5457	0.2656	0.5312	[1.0145; 2.0769]
PBMCs + ciMSCs treated donor	1.6651	0.2656	0.5312	[1.1339; 2.1963]
PBMCs + ciMSCs unrelated donor	1.461	0.2656	0.5312	[0.9298; 1.9922]

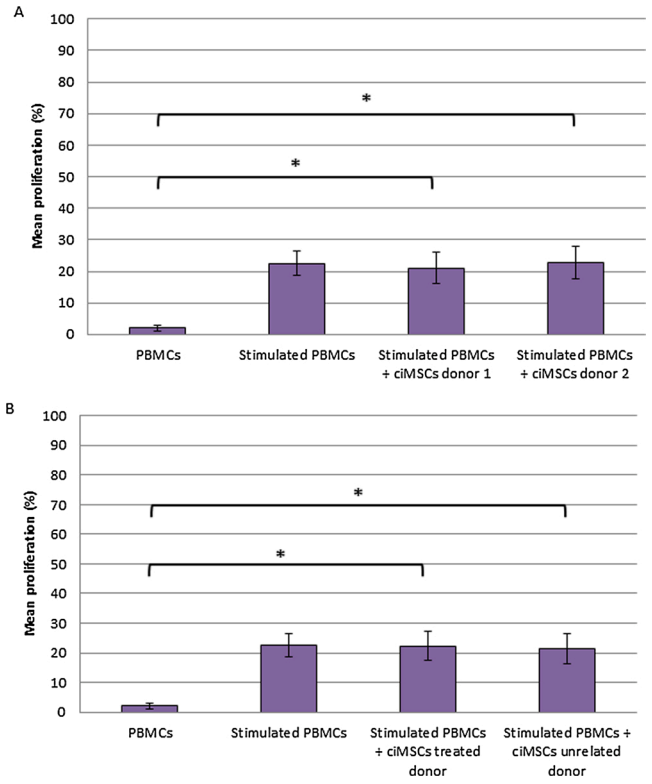


Fig. 5. Mixed lymphocyte reaction assay; Immunomodulation of allogeneic ciMSCs. A. Allogeneic ciMSCs from two different donor horses cocultured with stimulated PBMCs derived from horses having received at least 3 ciMSC treatments derived from one of these two donors. **B.** Allogeneic ciMSCs cocultured with stimulated PBMCs derived from horses having received at least 3 treatments of the same donor. Bars indicate the mean proliferation percentage ± SD. * $P < 0.0125$. PBMCs: peripheral blood mononuclear cells; ciMSCs: chondrogenic induced mesenchymal stem cells.

Table 3
Calculations of the 95 % confidence intervals of the treatment groups as compared to the positive control.

Treatment	Estimate	SE	2*SE	95 % CI
PBMCs + ciMSCs donor 1	−0.9593	0.697	1.394	[−2.3533; 0.4347]
PBMCs + ciMSCs donor 2	0.3557	0.697	1.394	[−1.0383; 1.7497]
PBMCs + ciMSCs treated donor	0.1167	0.697	1.394	[−1.2773; 1.5107]
PBMCs + ciMSCs unrelated donor	−0.7203	0.697	1.394	[−2.1143; 0.6737]

incubation with serum derived from repeatedly treated horses (3.76 %) in comparison to the positive control (99.6 %) ($P < 0.001$) (Fig. 7 and Supplementary Fig. 5).

6.8. Anti-BSA antibodies

The serum of ten jumping and dressage horses was available for the anti-BSA antibody determination by means of an ELISA assay. The threshold for presence of anti-BSA antibodies in the equine serum was defined as a fold increase > 2 compared to the negative control (Gershwin et al., 2012; Owens et al., 2016). Seven of the ten jumping and dressage horses had anti-BSA antibody titers ranging from 3 to 16 times higher than the negative control (Fig. 8). When comparing these results with the joint effusion scores, 20 % of the horses (n = 2) had a higher effusion score of 1 or 2 while in 70 % of the horses higher

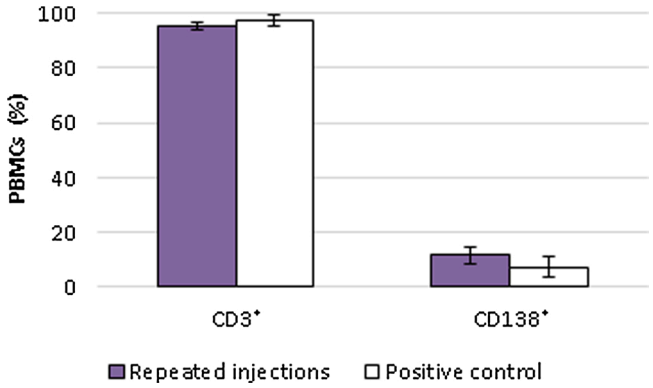


Fig. 6. Percentage of $CD3^+$ (T-lymphocytes) and $CD138^+$ (plasma cells) cells in the proliferated PBMC populations. The subsets of T-lymphocytes and plasma cells were determined in the proliferated group of stimulated PBMCs (positive control) and stimulated PBMC:ciMSC cocultures (repeated injections) of the mixed lymphocyte reaction for immunomodulation.

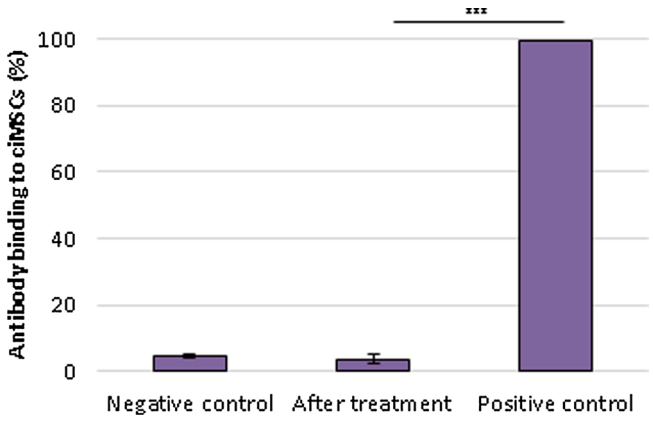


Fig. 7. Flow cytometric crossmatch assay after repeated injections with allogeneic ciMSCs. The negative control sample consisted of ciMSCs incubated with blanco serum (*i.e.* serum of a horse never treated with MSCs). This sample was used to determine background binding of the equine serum to the ciMSC and displayed a mean percentage of positive cells of 4.72 %. The positive control sample consisted of Aa^+ RBCs incubated with Aa- antiserum, resulting in a high positivity of 99.6 %. The test samples consisted of ciMSCs incubated with recipient serum, resulting in a mean percentage positive cells of 3.76 %. Bars indicate the mean percentage positive cells ± SD. *** $P < 0.001$.

anti-BSA antibodies were found. Therefore, no correlation between the anti-BSA antibodies and the effusion scoring was found in current study.

7. Discussion

Overall, no indications were found of a clinically relevant immune response directed against the ciMSCs. This was also reported in a comparable study investigating the effectiveness and safety of a repeated administration of allogeneic primed MSCs in an equine OA model (Barrachina et al., 2018). In the current study, in general, a decrease in joint effusion was observed after repeated administration of the stem cells, with similar evolutions for the parameters skin surface temperature, pain to palpation and the range of motion of the injected joints. One out of ten horses displayed an increase in joint effusion after the second treatment only. This adverse event is, however, commonly reported after (single) intra-articular administration of both MSC of different sources and saline solution and are generally self limiting (Caron, 2011; Carrade et al., 2011; Pigott et al., 2013; Joswig et al., 2017; Broeckx et al., 2018). Ardanaz et al. reported a temporary inflammatory response in healthy horses after an intra-articular injection

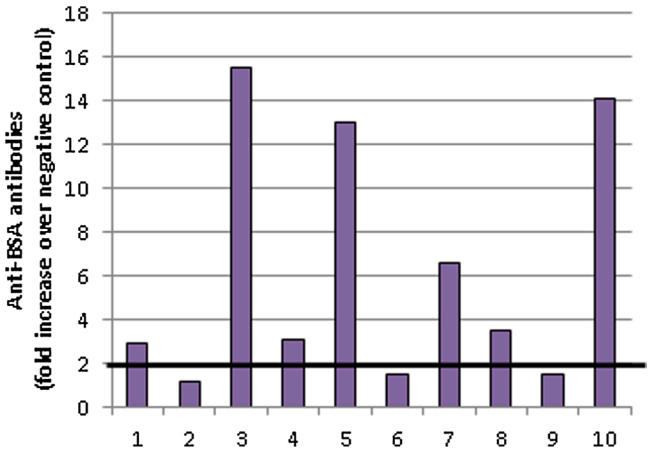


Fig. 8. Anti-BSA antibodies in horses after ciMSC administration. Seven of the ten horses (70 %) had a positive anti-BSA antibody titer (fold increase > 2 compared to the negative control). Three of the ten horses were negative for anti-BSA antibodies (this threshold is defined as a fold increase < 2 when compared to the negative control).

with autologous and allogeneic MSCs (Ardanaz et al., 2016). Additionally, a mild synovial effusion and transient increase of white blood cell count, neutrophil count and total protein in the synovial fluid was observed. However, in the present study no synovial fluid was sampled since mild synovitis can be caused by repeated arthrocentesis (Caron, 2011). The clinical signs observed in the present study were thus in line with previously performed experiments supporting the assumption that allogeneic ciMSCs are a safe treatment option even after repeated intra-articular administration from the clinical point of view.

Furthermore, immunogenicity of the ciMSCs was investigated in a modified one-way MLR. In this MLR, the recipients PBMCs were stimulated with ConA for the positive control according to previously published methods (Colbath et al., 2017; Ranera et al., 2017), instead of a coculture with donor and recipient PBMCs. After coculture of PBMCs from repeatedly treated horses with allogeneic ciMSCs, the ciMSCs did not induce a cellular immune response as demonstrated by the stable PBMC proliferation rates compared to the negative control. These results are independent of the fact that the ciMSC originate from the same donor horse as used for treatment or an unrelated donor. In a comparable study, the immunogenicity of autologous and allogeneic BM-MSCs was investigated (Colbath et al., 2017). It was observed that the allogeneic MSCs did not generate a strong alloreactive T cell response. Moreover, the observed small degree of T cell activation was equal to the response generated by autologous MSCs. Additionally, Carrade et al. (2011) reported a similar response of healthy equine joints to a single intra-articular injection of autologous and allogeneic placenta-derived MSCs. Indeed, there were no significant differences between the inflammatory response induced by autologous and allogeneic MSCs. Furthermore, Pigott et al. (2013) re-exposed PBMCs to autologous, allogeneic and xenogenic BM-MSCs after a single intra-articular injection. This resulted in an increase of T-lymphocyte proliferation upon re-exposure to xenogeneic MSCs, but this was not seen in the autologous and allogeneic treatment groups. However, it is important to take under consideration that the tissue source for MSC isolation is of great importance. For example, BM-MSCs are heterogeneous in MHC II expression (Schnabel et al., 2014), so that these MSCs as well as the patients receiving the cells should always be tested for MHC expression to detect a MHC mismatch before clinical application.

There are certain parameters that can influence the MHC expression. In a recent study, MSCs were pre-treated with TGF-β and a subsequent reduced MHC expression was observed (Berglund and Schnabel, 2017). TGF-β is one of the cartilage stimulating growth factors used to chondrogenically predifferentiate the MSCs, hence this might explain the very low immunogenicity of ciMSCs reported in the repeatedly treated horses in the present study. Additionally, the MHC I expression on PB-derived MSCs is below 10 % (Broeckx et al., 2014b), which could be another reason for the limited induction of the cellular immune response seen by our group. In previous studies performed by our group, patients were not MHC matched with donor MSCs (Beerts et al., 2017; Broeckx et al., 2014a, 2014b; Broeckx et al., 2018, 2019a, 2019b; Depuydt et al., 2021; Vandenberghe et al., 2015) and similarly no clinical problems were detected.

The immunomodulatory properties of ciMSCs were also investigated in the present study using a modified one-way MLR. The coculture of allogeneic equine ciMSCs with stimulated PBMCs from repeatedly treated horses neither resulted in immunostimulation or immunosuppression. Indeed, there was no significant difference between mean PBMC proliferation in the positive control and the coculture groups. Comparable studies, albeit with untreated horses, report an immunosuppressive effect of equine MSCs *in vitro* (Carrade et al., 2012; Ranera et al., 2016; Colbath et al., 2017). Carrade et al. (2012) showed that equine MSCs derived from bone marrow, adipose tissue or cord blood reduced T-cell proliferation after stimulation and Colbath et al. (2017) reported that the addition of autologous and allogeneic BM-MSCs elicit an equal suppression of PBMC proliferation. Additionally, Ranera et al. (2016) reported that equine allogeneic BM-MSCs suppress the proliferation of mismatched PBMCs.

The absence of the immunomodulatory effect of ciMSCs in the current study can perhaps be ascribed to the chondrogenic induction of MSCs (Lohan et al., 2014; Ryan et al., 2014). As a result of this predifferentiation, the anti-inflammatory properties of MSCs could possibly evolve into more chondrogenic capacities, aiding a more durable structural healing of the cartilage (Murphy et al., 2015; Spaas et al., 2015). This mode of action might be more effective than a pure immunomodulatory function for the current indication, since previous work reported only a modest improvement of osteoarthritis treated with bone-marrow derived MSCs, what dampened the enthusiasm of the use of native MSCs (Frisbie et al., 2009). This observation gave rise to the hypothesis that chondrogenic induction of MSCs may provide more promising results. Literature reports successful *in vitro* chondrogenesis of MSCs when chondrogenic inductive growth factors are used (Indrawattana et al., 2004; Mauck et al., 2006; Handorf and Li, 2011; Chen et al., 2013; Gardner et al., 2013; Murphy et al., 2015; Desance et al., 2018).

Additionally, *in vitro* observations have confirmed a more durable cartilage quality with chondrogenic induced MSCs compared to native MSCs (Spaas et al., 2015). On top of that, a recent study reported that an inflammatory synovial environment does not promote immunoregulatory changes in BM-MSCs (Barrachina et al., 2016). This implies that the inflammatory setting in an injured joint might not be adequate enough to activate the immunosuppressive potential of native BM-MSCs or MSCs from other sources. Therefore, the authors suggested priming BM-MSCs with specific factors in order to obtain a better outcome. These *in vitro* results indicate that chondrogenic induced MSCs are safe to be used in an *in vivo* setting. As a matter of fact, *in vivo* studies with intra-articular application of ciMSCs have been performed by our group, without any (serious) adverse events or suspected adverse drug reactions (Broeckx et al., 2014a, b; Broeckx et al., 2018). Moreover, clinical improvement and a chondroprotective effect of ciMSCs in an osteoarthritis model was observed, together with clinical improvement and higher rate or returning to work in a field study on patients (Broeckx et al., 2014a, b).

The flow cytometric crossmatch assay revealed no presence of allo-ciMSC antibodies. Thus, indicating no humoral immune response development after repeated intra-articular injections with ciMSCs. In contrast, studies from other research groups reported the development of anti-MSC antibodies in the horses after single or repeated MSC administration (Owens et al., 2016; Barrachina et al., 2020). However, Barrachina et al. (2020) reported only allo-antibody production in case of an MHC halfmatch and mismatch with the donor horse using a

microcytotoxicity assay that allows detection of antibody reactions specifically directed against MHC. Nevertheless, in the study of Owens et al. (2016), MSCs were derived from bone marrow (BM) or adipose tissue (AT) being reported to have high expression of MHC I (Schnabel et al., 2014; Tan et al., 2017) and for BM even a high expression of MHC II is possible (Schnabel et al., 2014), which increases the risk for immune response development. Furthermore, the different dosages of MSCs used for this study (25–80 million MSCs per treatment) were very high compared to the amount of ciMSCs (1.4–2.5 million per treatment) used by our group, what also may increase the rate of antibody development.

Literature reports that alloantibodies can develop in response to exposure to FBS. Annual vaccination of horses with vaccines that are made in media containing FBS can result in the development of antibodies directed against BSA, which is the major component of FBS (Sundin et al., 2007; Gershwin et al., 2012). The cell culture medium used for the cultivation of ciMSCs applied in this study was also supplemented with 20 % FBS. Therefore, it is important to investigate whether antibodies were developed directly against the ciMSCs or against the FBS in which the ciMSCs are cultivated. The ELISA assay revealed presence of anti-BSA antibodies in seven of the ten horses. This result was in line with a study from another group where 89 % of the horses were positive for anti-BSA antibodies before and after allogeneic MSC treatment (Owens et al., 2016). The present study shows that an allogeneic ciMSC treatment does not induce antibodies directed against the MSCs, even if anti-BSA antibodies are present.

Because the horses were not treated all at the same time, the period between the last treatment administration and the peripheral blood collection for the MLR and flow cytometry crossmatch assays varied between the recipient horses, which could have affected the results of these assays regarding the detection of a cellular and or humoral immune response after re-exposure. However, to reduce inter-test variation, the blood collection of the horses was taken on a single occasion, which means that the interval between last treatment and blood sampling was very variable. According to a placebo-controlled clinical trial where human Crohn patients were treated one time with allogeneic adipose-derived MSCs or placebo, donor-specific antibodies were detected in 34 % of the patients treated with MSCs and none were detected in placebo-treated patients at week 12 (Panes et al., 2016). In this case, donor-specific antibodies were detected three months even after a single injection of allogeneic mesenchymal stem cells. Additionally, a recently published article reports no detection of alloantibodies at different time points following allogeneic MSCs treatment for osteoarthritis in dogs (4 weeks, 12 weeks and 24 weeks post-injection) (Cabon et al., 2019). This also confirms that a 12 week (or 3 month) time point following treatment administration is relevant. On top of that, Vujanic et al. (2012) reported higher IgG levels in the recipient's serum at 1, 3, 6, and 12 months postimmunization. Sheep received three immunizations which were separated by 3 weeks. This was however in case of immunization using vaccines against influenza viruses, which is another indication then allogeneic MSC treatment, yet also indicates the relevance of immunogenic investigation at 3 months after repeated immunization.

A second limitation of the current study is that no serum samples were available before treatment administration, so no changes in alloantibody or anti-BSA antibody responses could be evaluated. However, as the study was performed on field patients retrospectively, blood collection on fixed time points was restricted due to practical considerations.

Due to the retrospective study design, lameness scores were not available for all included horses and could therefore not be taken under consideration. However, at least six of these horses completed successfully in international shows following the last injections.

Finally, the MHC haplotype from both donor and recipient horses was unknown. It has been reported that anti-MHC antibodies are produced when donor and recipient are MHC mismatched (Barrachina et al., 2020). Since a very high level of MHC heterozygosity and haplotype diversity has been reported (Holmes et al., 2019; Miller et al., 2017), it seems unlikely that all the included horses and donor horses would possess identical MHC haplotypes. Even though other previous studies investigating alloantibodies also did not take the MHC haplotypes into account (Owens et al., 2016), the current results should be taken under consideration before extrapolation.

8. Conclusion

No serious clinical adverse reactions or suspected drug reactions were found following repeated intra-articular administration in horses. Furthermore, this retrospective study indicates no cellular or humoral immune response is induced in horses repeatedly treated with chondrogenic induced equine allogeneic peripheral blood-derived MSCs.

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Author contributions statement

Authors JHS, LVH and CM conceived the study and planned the design. Clinical assessment of the horses was performed by MS. Mixed lymphocyte reactions and alloantibody assays were performed by LVH and EDP. LDD and MP performed the anti-BSA antibody assay. Statistical analysis was done by LD. LVH, CM and EDP wrote the first draft of the manuscript. JHS, SYB, CB and FG provided supervision and critical review of the manuscript. All authors contributed and approved the final version of the manuscript.

Declaration of Competing Interest

The author JHS declares competing financial interests as shareholder in GST at the time of the study. SYB, JHS, ED, CB, and LVH are all employed by GST at the time of the study. JHS is an inventor of a pending patent covering immunomodulating technology owned by GST. The content of this manuscript contains the registered stem cell based medicinal product Arti-Cell® Forte owned by GST and patents related to this product. Authors CM and MS are employed by the company Veterinary Innovation Alliance CV. Author MS is employed by the company Suls Equine Veterinary Services BV. The remaining authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.vetimm.2021.110306.

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