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ORIGINAL ARTICLE

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Equine allogeneic tenogenic primed mesenchymal stem cells: A clinical field study in horses suffering from naturally occurring superficial digital flexor tendon and suspensory ligament injuries

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Abstract

Background: Mesenchymal stem cells are an innovative therapeutic for various equine orthopaedic diseases, including soft tissue injuries.

Objectives: To evaluate the safety and efficacy of tenogenic primed equine allogeneic peripheral blood-derived mesenchymal stem cells (tpMSCs) in horses with naturally occurring superficial digital flexor tendon (SDFT) and suspensory ligament (SL) injuries.

Study design: Multicentre, blinded, randomised, placebo-controlled clinical trial.

Methods: One hundred client-owned horses with SDFT and SL injuries were randomised to receive an intralesional tpMSC (66) or saline (34) injection. Clinical and ultrasonographic evaluation was performed before treatment and on Days 56 ± 3 and 112 ± 3 after treatment. Long-term data on re-injury was collected up to 2 years after treatment.

Results: Significantly more tpMSC-treated horses achieved improvement in fibre alignment score (FAS) (100% vs. 54.5%, $p < 0.001$) and echogenicity (97.0% vs. 57.6%, $p < 0.001$) on Day 112 ± 3, and their lesion size decreased significantly (-27.6 ± 25.91 vs. -4.6 ± 26.64 mm^2, $p < 0.001$) compared to the placebo group. A FAS = 0 was achieved in 65% of tpMSC-treated horses, as compared to 9% of placebo-treated horses at Day 112 ± 3. The attending veterinarians reported no re-injury in 41 of 53 tpMSC and in 2 of 26 saline-treated horses available for long-term follow-up ($p < 0.001$).

Main limitations: As this study consisted of client-owned horses, no samples for histology were collected. Long-term follow-up was only available for a subset of enrolled horses.

Conclusions: The intralesional administration of tpMSCs was safe and improved the quality of healing and long-term outcomes in sports horses with naturally occurring SDFT and suspensory injuries.

Stephanie Carlier and Eva Depuydt contributed equally to this study.

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KEYWORDS
desmitis, horse, stem cell therapy, tendonitis

1 | INTRODUCTION

Injuries of tendons and ligaments are among the most common orthopaedic injuries in sports horses, often compromising or ending their athletic career.[1–3] Tendon and ligaments tend to heal with the formation of scar tissue, often leading to reduced functional capacity of these structures and a substantial risk of re-injury.[4] The need to restore tendon functionality has prompted more research into treatment modalities that could improve the healing process, such as autologous and allogeneic orthobiologics, including stem cell therapies.[5] Studies investigating the efficacy of autologous bone marrow-derived mesenchymal stem cells (BM-MSCs) and, to a lesser extent, adipose tissue-derived MSCs (AT-MSCs) in tendinopathies have reported successful results.[6,7] To date, available data on efficacy of autologous BM-MSCs and AT-MSCs and tenogenic primed equine allogeneic peripheral blood-derived mesenchymal stem cells (tpMSCS) in naturally occurring disease show promising results, but the studies either lack a control group,[6,8] or comprise only a limited number of animals per group.[9,10] TpMSCs represent a convenient treatment option as they are ready to use and consistently manufactured according to rigorous standards. To investigate the safety and efficacy of tpMSCs for the treatment of superficial digital flexor tendon (SDFT) and suspensory ligament (SL) lesions, a placebo-controlled, blinded field trial was performed in 100 sport horses.

2 | MATERIALS AND METHODS

2.1 | Study design and horses

This was a multicentre, blinded, placebo-controlled and randomised clinical trial with a duration of 112 ± 3 days, evaluating the efficacy of tpMSCs. Each horse met the inclusion criteria before enrollment (Table 1). Based on the recorded duration (< or ≥2 weeks duration) and ultrasonographic appearance, the lesions were further divided into three categories: (1) acute (<2 weeks duration), (2) subacute (≥2 weeks duration), (3) chronic (≥2 weeks duration with the presence of chronic changes on ultrasound, e.g., heterogeneous echo, presence of fibrotic tissue or enthesiophytes). In addition, the severity based on the cross-sectional area (CSA) was graded retrospectively according to available literature.[11] The CSA of the lesion of <20%, 20%–35% and >35% was considered mild, moderate and severe, respectively.

Additional long-term follow-up data was collected on the incidence of re-injury for up to 2 years from the time of treatment.

To ensure masking, separate personnel were used for clinical examinations and for treatment administration. To establish consistency, for each horse, the examinations involved the same experienced handler, surfaces and investigator throughout the entire study.

TABLE 1 Inclusion criteria.

Inclusion criteria
Signed owner consent
First-time unilateral SDFT or SL lesion by overstrain injury
Changes on ultrasound
Clearly defined anechoic or hypoechoic lesion occupying >10% of the cross-sectional area (CSA) of the tendon at the maximum injury zone (MIZ)
49% or less parallel fibre bundles in the lesion
Moderate to severe hypoechogenicity (the lesion is 50% or more anechoic)
An increase in anterior–posterior thickness (APT) of ≥0.1 cm compared to the contralateral tendon at the MIZ
Combined sum score of ≥3 had to be present based on tendon swelling, pain to pressure heat and lameness (American Association of Equine Practitioners, AAEP) score (Table 2).

2.2 | Investigational product and control product

The investigational product (IVP), RenuTend®, consisted of a proprietary formulation of tpMSCs. The donor qualification process and the manufacturing were performed under the same high standards as previously described.[12–14] The samples were cryopreserved at −80°C until further use. In the present study, two tpMSC batches derived from two different donor horses were used. The control product (CP) consisted of 1 mL sterile saline injectable solution (0.9% NaCl).

2.3 | Treatment administration, randomisation, assessments and exercise scheme

At Day 0, after assessing baseline clinical and ultrasonographic parameters, each horse was sedated with detomidine hydrochloride (20 µg/kg bodyweight) and the area around the injection site was clipped and aseptically prepared. Next, the IVP or CP was administered by ultrasound-guided intralesional injection in the affected SDFT or SL. The random treatment allocation plan was created using MS Excel 2013 (Microsoft) with a block size of 3 and a 2 IVP:1 CP allocation. A list with the treatment allocation per patient number was prepared by the statistician based on the different permutations. No ancillary therapies (e.g., shockwave, Class 4 laser) or concomitant treatments (other than the single NSAID injection on Day 0) were allowed during the rehabilitation phase up to Day 112 ± 3.

Assessments were performed according to the study protocol (Figure 1). In addition to the physical assessment, all horses were observed daily by the caretaker for abnormal clinical signs, including the potential occurrence of any apparent abnormal tissue formation. Other clinical assessments were scored as described in Table 2. Tendon assessment consisted of an assessment of swelling, pain to pressure and heat and circumference of the treated tendon/ligament

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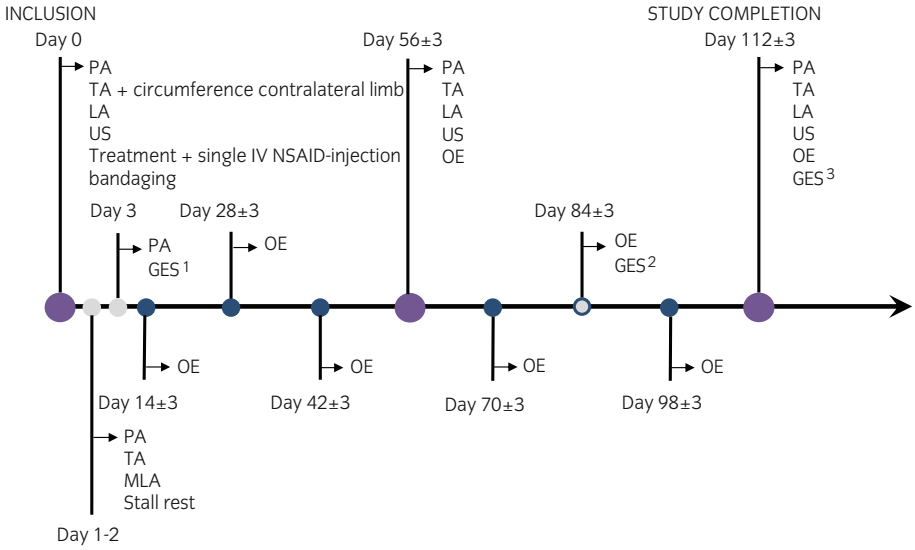

FIGURE 1 Study protocol. GES, gradual exercise schedule; GES[1], start hand walking; GES[2], incremental increase of walking and trotting under the rider, if there was ultrasonographic improvement on day 56 ± 3; GES[3], start canter work, provided FAS and ES = 0 and lesion CSA <5% tendon CSA; LA, lameness assessment; MLA, modified lameness assessment; OE, owner evaluation; PA, physical assessment; TA, tendon assessment; US, ultrasound.

(measured using measuring tape at the maximum injury zone (MIZ) with the limb in a weight-bearing position). The circumference of the contralateral limb was measured at the same location on Day 0 only. The ultrasound examination was performed using a 5–12 MHz linear Array T-probe (with a stand-off pad). The tendon/ligament of the contralateral limb was examined on Day 0 only. During this evaluation, fibre alignment score (FAS), echogenicity score (ES), lesion size (CSA: mm^2 and % of the tendon), and anterior–posterior thickness (APT) were evaluated at the MIZ (Table 2). Any ultrasonographic presence of abnormal tissue formation was recorded.

The owner evaluation included the improvement and working status of the included horses (Table 2).

Starting on Day 3, the horses were hand-walked three times daily. If ultrasonographic improvement was observed on Day 56 ± 3, an incremental progression of trotting was prescribed based on clinical and ultrasonographic improvement throughout the study, starting Day 85 post-injection. If there was no ultrasonographic improvement on Day 56 ± 3, the horses were continued three times daily hand walking (Table S1).

2.4 | Long-term follow-up

Additional data was recorded via a questionnaire (Data S1) completed by the investigating veterinarians following study completion for up to 2 years from the time of treatment, regarding the re-injury of the same tendon/ligament or any apparent abnormal tissue formation. Horses available for long-term follow-up were still under the management of the respective investigating veterinarian.

3 | DATA ANALYSIS

All statistical analyses, except for the statistical analysis of the long-term re-injury data, were performed using SAS® statistical analysis software version 9.4. The sample size was assessed using a two group χ^2 test with a 0.050 two-sided significance level and was calculated to have 80% power to detect the difference between a CP proportion, π_1, of 0.3 and an IVP proportion, π_2, of 0.6 or 0.65 for sample sizes of 23–31 and 46–62, respectively (a total sample size of 69–93). Considering a drop-out rate of approximately 7%, at least 100 (≈ 66 in IVP and ≈ 33 in CP) animals had to be recruited into the study. Summary statistics included the number and percent of each score by treatment group and day of observation for FAS, ES, tendon and lameness assessments, owner's opinion regarding improvement and working status. The treatment groups were compared to each other and for change from baseline using the Mantel–Haenszel test for FAS, ES and tendon and lameness assessment. Groups were compared using the Mantel–Haenszel test for the owner's opinion regarding improvement and working status after Day 112 ± 3. Number of concomitant treatments per horse after day 112 is not discussed in this paper. Groups were compared for change from baseline using the Wilcoxon test for lesion size (mm^2 and % of CSA of the tendon), increase of tendon circumference and ectopic tissue formation (until Day 112 ± 3). Summary statistics for lesion size and tendon circumference were prepared by treatment group and day of observation. Summary statistics for the formation of ectopic tissue were performed by the treatment group for Days 56 ± 3 and 112 ± 3. Groups were compared using Fisher's exact test for relevant improvement based on the FAS (FAS = 0) on Day 112 ± 3 and the incidence of adverse events (AEs). A chi-square test (using GraphPad Prism version 9.5.1. for MacOS, GraphPad Software) was used to assess the difference in the re-injury rate between both groups. A 5% significance level ($p < 0.05$ for two-sided tests) was used to assess statistical differences (respectively, 2.5% if one-sided tests were used). From a clinical perspective, relevant improvement was based on the FAS on Day 112 ± 3 as the most relevant parameter for the target disease. All other p values should be interpreted descriptively.

4 | RESULTS

4.1 | Horses

A total of 100 client-owned horses in active sport or training before injury were enrolled (Table 3). All but one horse completed the study

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TABLE 2 Clinical scores of veterinary assessments including lameness, modified lameness assessment, local heat, swelling and pain to pressure, fibre alignment score (FAS) and echogenicity score (ES) and owner assessment of the horse's improvement and work status.

Veterinary assessments	Score system	Definition
Modified lameness assessment scoring (walk up and down on a straight line)	0	Lameness not perceptible
	1	Lameness is difficult to observe and is not consistently apparent, but visible
	2	Lameness is obvious at a walk
	3	Lameness produces minimal weight bearing in motion and/or at rest or a complete inability to move
Lameness assessment (AAEP lameness scale)	0	Not perceptible under any circumstances
	1	Difficult to observe and is not consistently apparent, regardless of circumstances
	2	Difficult to observe at a walk, or when trotting in a straight line, but consistently apparent under certain circumstances
	3	Consistently observable at a trot under all circumstances
	4	Obvious at a walk
	5	Minimal weight bearing in motion and/or at rest or a complete inability to move
Tendon/ligament assessment: Heat	0	No increased temperature sensation
	1	Slightly increased temperature sensation
	2	Moderately increased temperature sensation
	3	Severely increased temperature sensation
Tendon/ligament assessment: Pain to pressure	0	No pain to pressure
	1	Slight pain to pressure
	2	Moderate pain to pressure
	3	Severe pain to pressure
Tendon/ligament assessment: Swelling	0	No swelling
	1	Slight swelling
	2	Moderate swelling
	3	Severe swelling
Fibre alignment score (FAS)[15]	0	≥75% parallel fibre bundles in the lesion
	1	50%–74% parallel fibre bundles in the lesion
	2	25%–49% parallel fibre bundles in the lesion
	3	≤25% parallel fibre bundles in the lesion
Echogenicity (ES)[15]	0	Normal echogenicity
	1	Mild hypoechogenicity, the lesion is slightly anechoic
	2	Moderate hypoechogenicity, 50% of the lesion is anechoic
	3	Severe hypoechogenicity, the lesion is mostly anechoic
Owner assessment		
Horse improvement	0%	No improvement
	20%	Marginal improvement
	40%	Mild improvement
	60%	Moderate improvement
	80%	Large improvement
Work status	Failure to return to work	
	Rehabilitating	
	Return to (lower level of) work	
	Return to previous or higher level of work	

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TABLE 3 Signalment of the included horses, including age, sex, breed, discipline, level, affected limb and location.

Parameter	Statistics/subdivisions	IVP ($n = 66$)	CP ($n = 34$)	Total ($n = 100$)
Age	Mean (SD)	11.8 (4.95)	12.6 (5.07)	12.1 (4.98)
	Min–Max	3–25	5–25	3–25
	Median	11.0	12.0	12.0
	Q1–Q3	9.0–14.0	9.0–15.0	9.0–15.0
Sex	Gelding	24 (36.4%)	15 (44.1%)	39 (39.0%)
	Mare	29 (43.9%)	15 (44.1%)	44 (44.0%)
	Stallion	13 (19.7%)	4 (11.8%)	17 (17.0%)
Breed	Arab	0 (0.0%)	1 (2.9%)	1 (1.0%)
	Horse (undefined breed)	2 (3.0%)	1 (2.9%)	3 (3.0%)
	Irish cob (tinker)	1 (1.5%)	2 (5.9%)	3 (3.0%)
	Lusitano	0 (0.0%)	1 (2.9%)	1 (1.0%)
	New Forest pony	1 (1.5%)	0 (0.0%)	1 (1.0%)
	Pinto	0 (0.0%)	1 (2.9%)	1 (1.0%)
	Pony (undefined breed)	3 (4.5%)	4 (11.8%)	7 (7.0%)
	Pura raza espanola (Andalusian)	1 (1.5%)	0 (0.0%)	1 (1.0%)
	Trotter	2 (3.0%)	2 (5.9%)	4 (4.0%)
	Warmblood	55 (83.3%)	22 (64.7%)	77 (77%)
Discipline	Dressage	22 (33.3%)	13 (38.2%)	35 (35.0%)
	Driving	0 (0.0%)	2 (5.9%)	2 (2.0%)
	Racing	0 (0.0%)	1 (2.9%)	1 (1.0%)
	Showjumping	39 (59.1%)	18 (52.9%)	57 (57.0%)
	Eventing	5 (7.6%)	0 (0.0%)	5 (5.0%)
Level	Training	29 (43.9%)	16 (47.1%)	45 (45.0%)
	Regional	6 (9.1%)	0 (0.0%)	6 (6.0%)
	National	9 (13.6%)	7 (20.6%)	16 (16.0%)
	International	22 (33.3%)	11 (32.4%)	33 (33.0%)
SDFT/SL affected	SDFT	29 (43.9%)	15 (44.1%)	44 (44.0%)
	SL	37 (56.1%)	19 (55.9%)	56 (56.0%)
Frontlimb/hindlimb affected	Frontlimb	51 (77.3%)	27 (79.4%)	78 (78.0%)
	Hindlimb	15 (22.7%)	7 (20.6%)	22 (22.0%)
Stage of injury	Acute/subacute	47 (71.2%)	26 (76.5%)	73 (73.0%)
	Chronic	19 (28.8%)	8 (23.5%)	27 (27.0%)
Seize of lesion	Mild (CSA <20%)	38 (57.6%)	17 (51.5%)	55 (55.5%)
	Moderate CSA (20%–35%)	23 (34.8%)	13 (39.4%)	36 (36.4%)
	Severe (CSA >35%)	5 (7.6%)	3 (9.1%)	8 (8.1%)

on Day 112 ± 3. The one horse, included in the CP group, was removed on Day 102 for reasons unrelated to the target disease or treatment.

4.2 | Treatment administration, randomisation and exercise scheme

At Day 0, all horses received a single intralesional administration in the affected SDFT or SL of either the IVP ($n = 66$) or CP ($n = 34$) according to the randomisation. Following treatment, an exercise regimen was prescribed based on clinical and ultrasonographic improvement throughout the study.

4.3 | Physical assessment

The percentage of horses having at least one AE (e.g., mild gastrointestinal problems and nasal discharge) was similar in both groups ($p = 0.7$) (Table S2). All but one horse fully recovered, and none of the AEs were regarded to be related to the study medication

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(neither CP nor IVP). One horse in the CP suffered from a severe AE, namely acquired myopathy on Day 102 during anaesthesia for a computed tomography examination and died during recovery. This AE was deemed unrelated to treatment, and the horse was removed from the study. Data collected from this horse before this AE was included in the statistical analysis (incl. Day 56 ± 3).

4.4 | Tendon assessment

Tendon assessment scores are presented in Figure 2. At Day 0, the scores for swelling, pain to pressure, and heat at the injection site were similar in both groups ($p = 0.6$; $p = 0.9$ and $p = 0.8$, respectively). Compared to baseline, the swelling decreased in significantly more horses in the IVP than in the CP group on Days 1 and 2 ($p = 0.002$ and $p = 0.01$, respectively). There was a decrease in swelling in 4.5% (3/66) and 10.6% (7/66) in the IVP group on Days 1 and 2, respectively, compared to 0.0% (0/34) on both timepoints in the CP group. In contrast, there was a higher increase in swelling in the CP group on Days 1 and 2, with 20.6% (7/34) and 11.8% (4/34), respectively. In the IVP group, this was 3.0% on both timepoints. Similarly, on Day 2, more horses in the IVP than the CP group showed a significant decrease in pain to pressure ($p = 0.03$), with 31.8% (21/66) of the IVP group and 11.8% (1/33) showing a decreased pain to pressure. At Day 56 ± 3 and 112 ± 3, respectively, the percentage of animals with a decrease in pain to pressure and swelling at the injection site, compared to baseline, was significantly higher in the IVP group compared to the CP group ($p < 0.001$), with more animals in the IVP group having no swelling ($p = 0.003$ and $p < 0.001$, respectively) and no pain ($p = 0.002$ and $p < 0.001$, respectively) (Figure 2A–C). On Day 112 ± 3, there was a decrease in pain in 93.9% (62/66) of the IVP group and 48.5% (16/33) of the CP group.

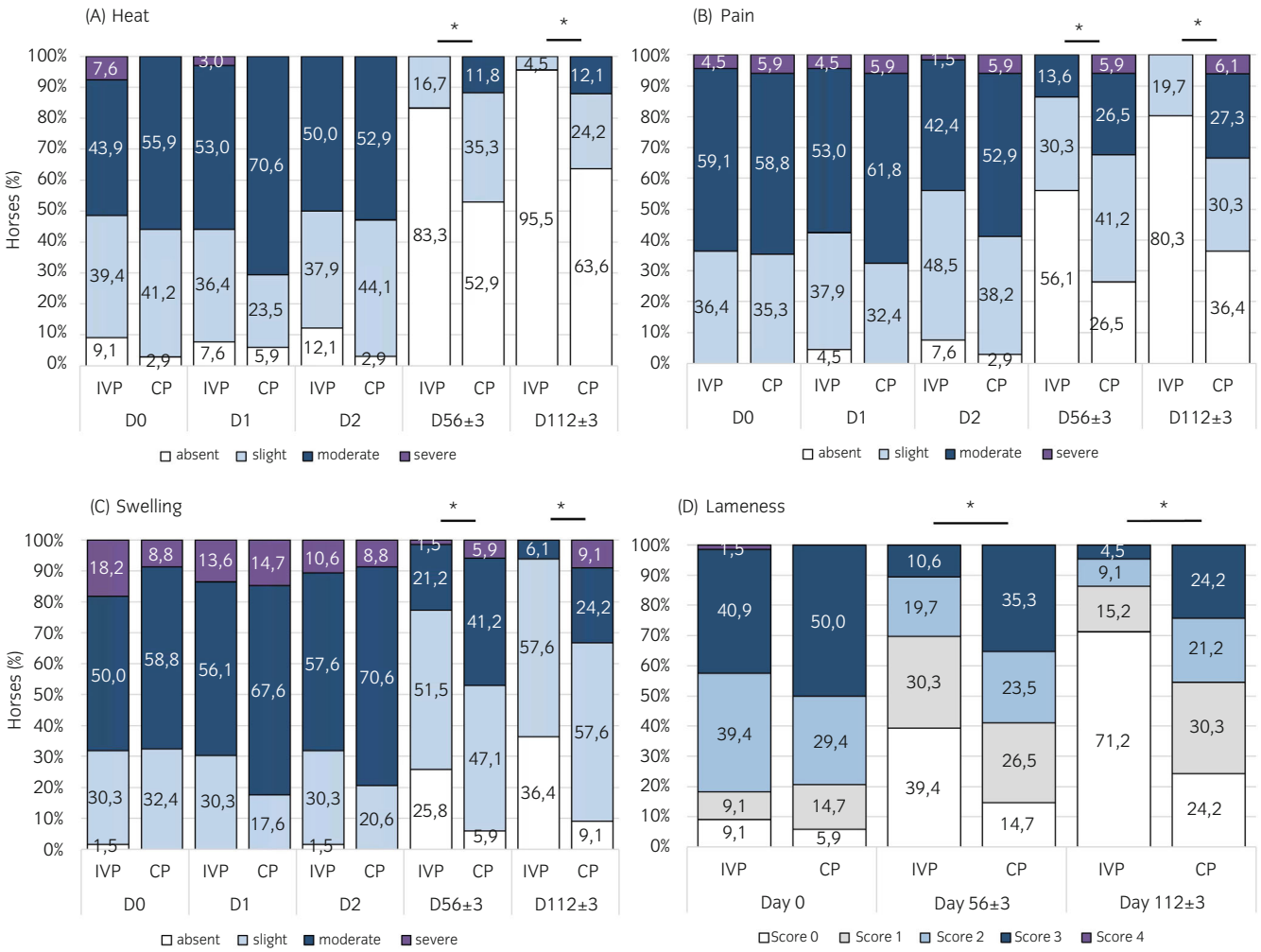

FIGURE 2 Proportion of scores per timepoint of (A) heat, (B) pain and (C) swelling and (D) lameness assessment (AAEP lameness scale). 0 = not perceptible under any circumstances; 1 = difficult to observe and is not consistently apparent, regardless of circumstances; 2 = difficult to observe at a walk, or when trotting in a straight line, but consistently apparent under certain circumstances; 3 = consistently observable at a trot under all circumstances; 4 = obvious at a walk; 5 = minimal weight bearing in motion and/or at rest or a complete inability to move. *$p \leq 0.05$, indicating a significant difference between the IVP group and CP group on that particular timepoint.

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At the same timepoint, the swelling had decreased in 80.3% (53/66) of the IVP-treated horses, compared to 39.4% (13/33) of the CP group.

On Day 1, there was a mean increase in the circumference of the affected limb in both groups; however, the increase was significantly higher in the CP group compared to the IVP group ($p = 0.02$) (0.07 ± 0.22 cm in the IVP groups vs. 0.13 ± 0.16 cm in the CP group). There was a significant difference in the circumference of the affected limb compared to baseline on both Day 56 ± 3 (−0.38 ± 0.62 vs. −0.11 ± 0.40 cm; $p = 0.006$) and Day 112 ± 3 (−0.69 ± 0.60 vs. −0.17 ± 0.44 cm; $p < 0.001$), with a higher decrease in circumference in the IVP group ($p = 0.006$ and $p < 0.001$, respectively).

No abnormal tissue formation was observed during ultrasonographic evaluation of the tendons or ligaments in any of the animals on any of the timepoints.

4.5 | Lameness assessment

Lameness scores are presented in Figure 2. On Day 0, the AAEP lameness scores were similar in both groups ($p = 0.7$). On Day 56 ± 3 and Day 112 ± 3, the percentage of horses having no perceptible lameness was significantly higher ($p < 0.001$) in the IVP group, compared to the CP group, as well as a significant reduction of lameness compared to the baseline ($p < 0.001$ and $p = 0.009$, respectively) (Figure 2D). There was no significant difference in the modified lameness assessment on Day 1 and 2 between both groups ($p = 0.3$ and $p = 0.5$, respectively).

4.6 | Ultrasonographic assessment

Ultrasonographic assessment scores are presented in Figure 3. FAS was equally distributed between both groups at Day 0 ($p = 0.6$). All horses in the IVP group achieved improved FAS, compared to 54.4% of the horses treated with CP at Day 112 ± 3 ($p < 0.001$). The FAS of the affected tendon/ligament was significantly different between groups on Days 56 ± 3 and 112 ± 3, with more horses in the IVP group achieving scores of 0 or 1 ($p < 0.001$). On Day 112 ± 3, a FAS of 0 was achieved in 65.2% of the IVP group, compared to only 9.1% of the CP group ($p < 0.001$) (Figures 3A, 4 and 5).

ESs were similar between groups at baseline (Day 0) ($p = 0.9$). At both recheck visits, a significantly higher percentage of horses in the IVP group had normal echogenicity or mild hypoechogenicity, whereas there were more scores of moderate and severe hypoechogenicity in the CP group ($p < 0.001$). Moreover, the percentage of horses with an improved echogenicity from baseline was significantly higher in the IVP group, both on Day 56 ± 3 and Day 112 ± 3 ($p < 0.001$) (Figure 3B).

Change in APT of the affected tendon/ligament was not significantly different between groups at any time point.

On Day 0, the horses in the IVP and CP groups had a lesion size of 40.5 ± 29.92 and 42.0 ± 33.84 mm^2, respectively. More horses in the IVP group achieved a decrease in CSA of the lesion from baseline than in the CP group, both on Day 56 ± 3 (−17.8 ± 22.07 vs. −5.1 ± 29.45 mm^2; $p < 0.001$) and Day 112 ± 3 (−27.6 ± 25.91 vs. −4.6 ± 26.64 mm^2; $p < 0.001$) (Figure 3C). Efficacy (FAS = 0 on Day 112 ± 3) could be achieved in mild, moderate and severe lesions (Figure 3D).

4.7 | Owner evaluation

The work status reported by horse owners is presented in Figure 6. There was a significant difference between both groups in the improvement of the horse observed by the owner on Days 28 ± 3, 42 ± 3, 70 ± 3, 84 ± 3, 98 ± 3 and 112 ± 3 ($p = 0.008$; $p = 0.005$; $p = 0.006$; $p = 0.02$ and $p < 0.001$, respectively), with more horses in the IVP group showing a mild to large improvement on Day 28 ± 3 and a large improvement to no discomfort noticeable on Days 42 ± 3, 70 ± 3, 84 ± 3, 98 ± 3 and 112 ± 3.

On Days 70 ± 3, 84 ± 3, 98 ± 3 and 112 ± 3, there was a significant difference between the IVP and CP groups ($p = 0.01$; $p = 0.008$; $p = 0.03$ and $p < 0.001$), with more horses in the IVP group returning to work at all follow-up time points. On Day 112 ± 3, 19.7% of the IVP-treated horses were reported to have returned to their previous level of work, compared to none of the CP horses. None of the IVP-treated horses had failed to return to work at study completion, compared to 45.5% of the CP-treated horses. (Figure 6A).

4.8 | Long-term follow-up

Long-term follow-up information was only available for horses that remained under the management of the investigating veterinarians ($n = 79$). The long-term data was available for 53 of 66 horses in the IVP group and 26 of 34 in the CP group. The average time of long-term follow-up was 2 years with range (16–36 months after injection). Twelve of the horses (12/53) that were treated with IVP and available for long-term follow-up were reported to have reinjured their affected tendon/ligament. Significantly more horses, available for long-term follow-up, reinjured in placebo versus RenuTend group (12/53 vs. 24/26, respectively, $p < 0.001$). The time to re-injury in the IVP group varied between 168 days and 2 years post-tpMSC administration and between 98 days (before study completion) and 16 months following intralesional saline injection in the CP group.

None of the horses were reported to have developed any external abnormal tissue formation during the long-term follow-up period of approximately 2 years. A comparison was made between the long-term re-injury rate and FAS = 0 at Day 112 ± 3. Horses that achieved FAS = 0 at the study conclusion were almost three times less likely to suffer re-injury in long-term follow-up. Seventy-eight percent of all the horses with a normalised FAS, regardless of the treatment group, did not suffer from re-injury at the time of long-term data collection, whereas horses without normalised fibre alignment had a re-injury rate of 61.7% (Figure 6B).

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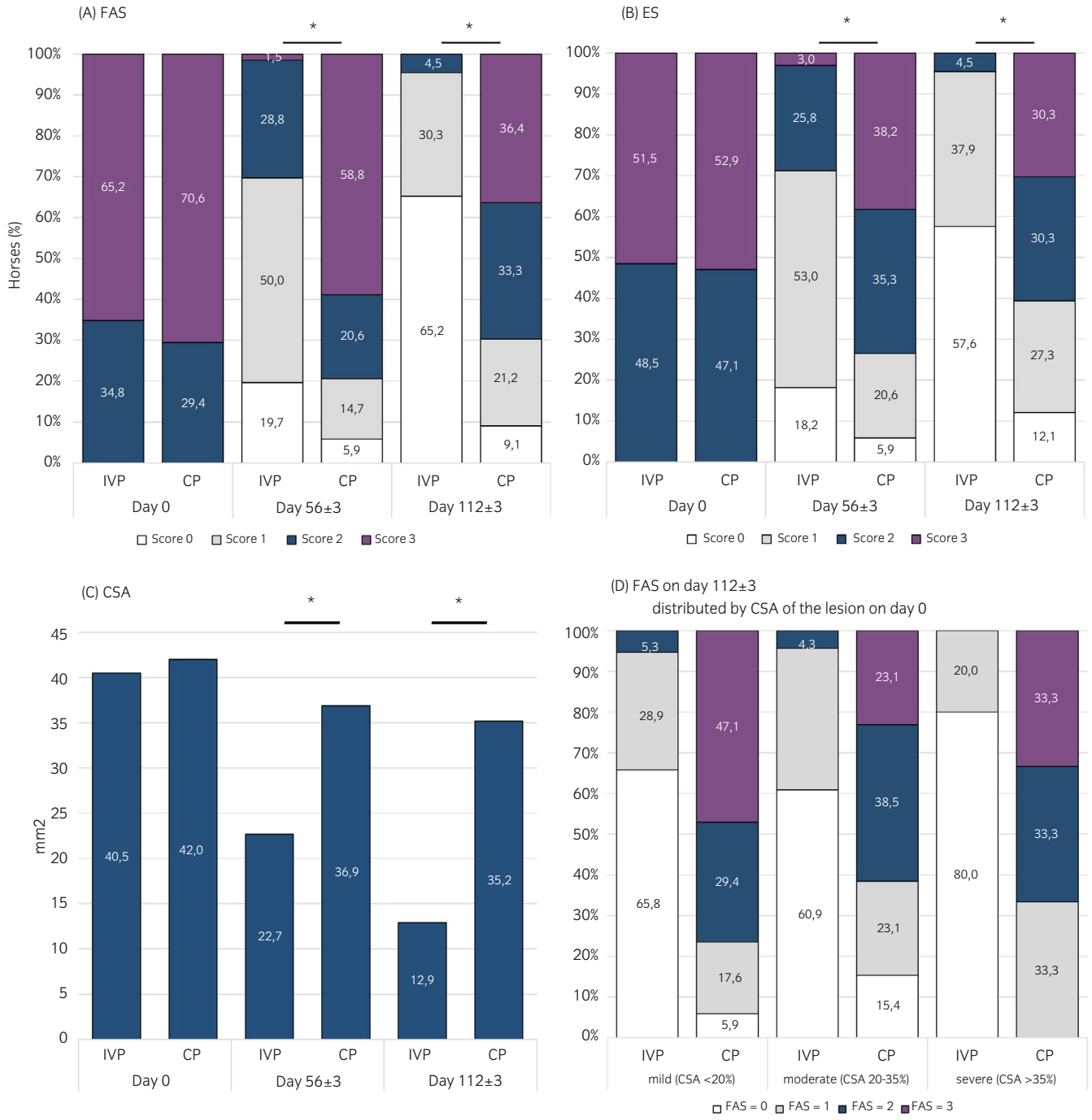

FIGURE 3 Ultrasound assessment. Proportion of scores per timepoint of (A) FAS: 0 = ≥75% parallel fibre bundles in the lesion, 1 = 50%–74% parallel fibre bundles in the lesion, 2 = 25%–49% parallel fibre bundles in the lesion and 3 = ≤25% parallel fibre bundles in the lesion, (B) ES: 0 = normal echogenicity; 1 = mild hypoechogenicity; 2 = moderate hypoechogenicity; 3 = severely hypoechogenicity and (C) CSA of the lesion (mm^2), (D) distribution of FAS scores on day 112 ± 3 based on the CSA of the injury on Day 0, respectively $^*p \leq 0.05$, indicating a significant difference between the IVP group and CP group on that particular timepoint.

5 | DISCUSSION

This placebo-controlled, randomised and blinded field study confirmed the safety and efficacy of tpMSCs (RenuTend®) as an intralesional treatment of naturally occurring SDFT and SL lesions in horses.

The safety of autologous and allogeneic stem cell products has recently been more of a focus due to increased use in musculoskeletal injuries in horses.[16] No suspected related drug reactions occurred following tpMSCS injection, as well as no abnormal tissue formation was observed in the tendons and ligaments following intralesional injection in any of the horses. Few studies have reported the prevalence of AEs following intralesional stem cell injection. One study reported no AEs following intralesional injection with autologous BM-MSCs,[10] and another study reported one AE in 230 treatments

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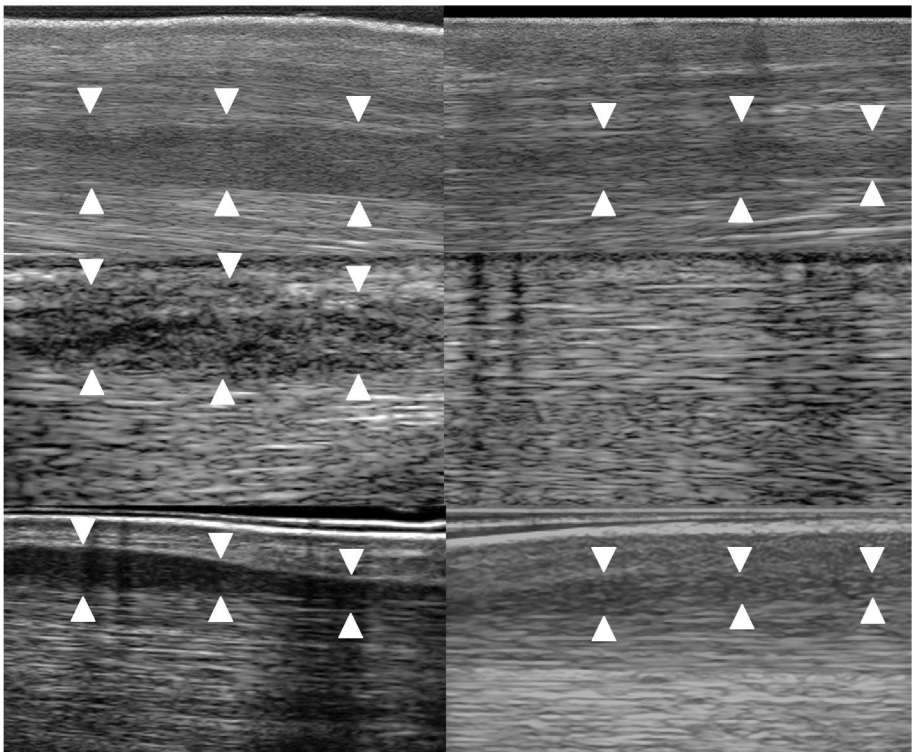

FIGURE 4 Longitudinal images of the SDFT on Day 0 (left) and Day 112 ± 3 (right) of three horses (A, B and C) treated with an intralesional injection of tpMSCs (IVP).

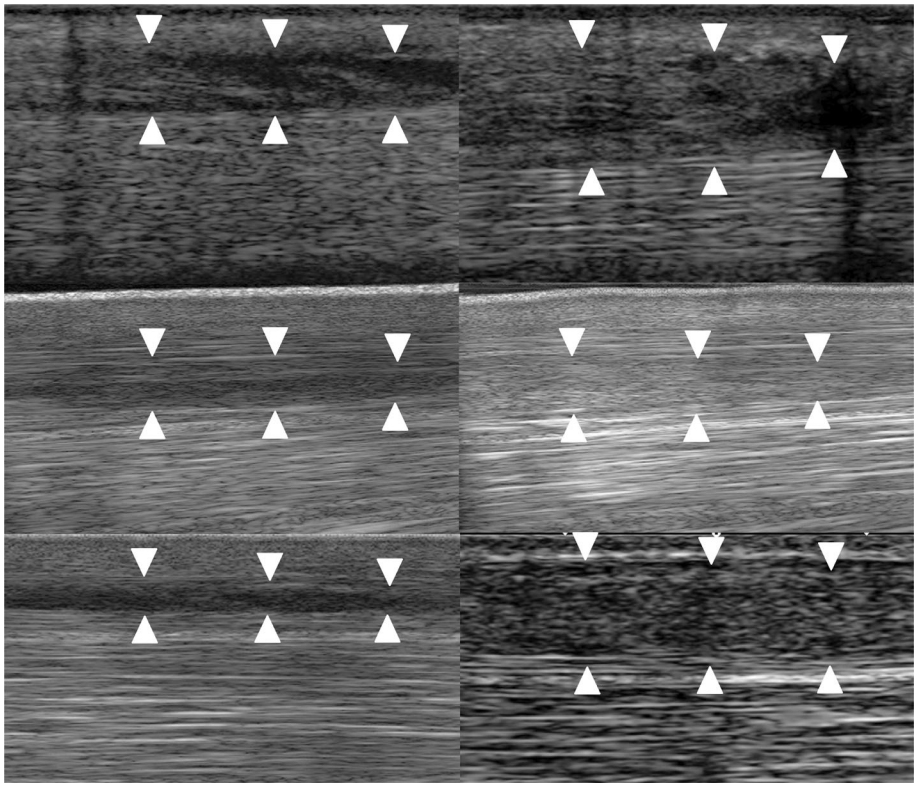

FIGURE 5 Longitudinal images of the SDFT on Day 0 (left) and Day 112 ± 3 (right) of three horses (A, B and C) treated with an intralesional injection of saline (CP).

involving increased lameness, likely due to compartmentalisation following swelling.[17]

Intralesional treatment with tpMSCs resulted in a faster decrease of swelling, pain to pressure, circumference at the level of injury and lameness compared to the control group. This could suggest an increased quality of healing with a decrease in tendon reactivity and size as compared to placebo.

Based on the results of the ultrasonographic examination, there was a significant improvement in FAS, ES and lesion size in the IVP group compared to the control group at all time points. In addition,

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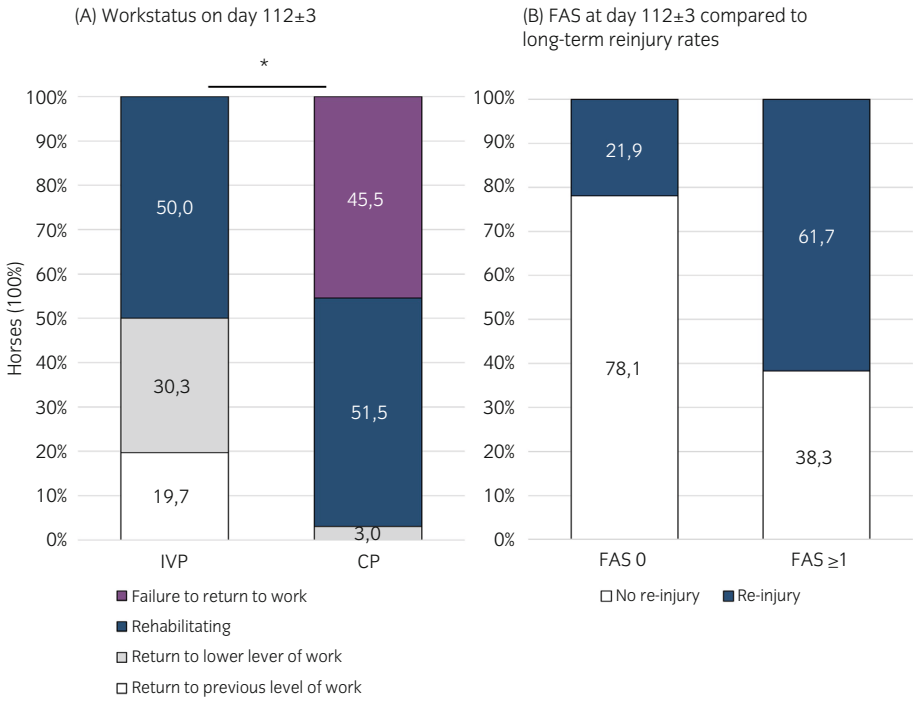

FIGURE 6 (A) Work status according to the owner on Day 112 ± 3. (B) FAS at day 112 ± 3 compared to long-term re-injury rates. *$p \leq 0.05$, indicating a significant difference between the IVP group and CP group on that particular timepoint.

65.2% of tpMSCs treated horses achieved (near to) normal fibre alignment by Day 112 (FAS = 0) as compared to only 9.1% in the placebo group. Studies using MSCs derived from amniotic and adipose tissue and bone marrow, have been performed and achieved similar results, with normalisation of FAS within 3–6 months following MSC injection.[18-20]

Moreover, the efficacy was seen in the different lesion sizes. This concurs with the hypothesis that the effective dose (number of administered tpMSCs) is not related to the size of the lesion. This agrees with previously reported data that there is no linear dose correlation with efficacy in stem cell therapy.[21,22]

Following study completion, additional long-term data was collected by the investigating veterinarian. A long-term success rate among horses available for follow-up was defined as lack of re-injury of the treated structure and was achieved in 77.4% of tpMSCs treated horses at 2-year follow-up ($p < 0.001$). This is much lower than re-injury rates reported for conventional treatments (43%)[23] or rehabilitation alone[24] and is similar to what has been reported for autologous BM-MSCs, where 23.7% of 141 horses were re-injured in a 2-year follow-up.[6] The re-injury rate in the placebo group in this study is higher than previously reported in untreated horses (up to 80%).[25] A possible reason for this discrepancy could be due to the loss of follow-up in 23.53% of the horses included in the control group, as this was not a part of the obligatory follow-up. It was also found that horses that achieved normalisation of fibre alignment at Day 112 (approximately 4 months after treatment) were nearly three times less likely to re-injure in the long-term as compared to horses with FAS ≥ 1, regardless of treatment group. This correlates to literature where FAS was found to serve as a good predictor of the final outcome and recurrence of the injury.[23] A FAS of 0 was only achieved in 3 out of 33 horses in the control group. Two of which were not available for long-term follow-up. As these horses would have higher odds of having a successful final outcome, the lack of available information on these horses could have a higher impact. In our study, significantly more horses treated with tpMSC achieved normalisation of FAS at 4 months post-treatment compared to the CP group. This, combined with low re-injury rates at 2-year follow-up, suggests that tpMSCs supported sustained healing of the tendon/ligaments. However, more research should be performed to confirm this.

A limitation of this study is that the assessment of the work status was based on the opinion of the owner. As such, this is largely dependent on the initial level of the horse and its expectations. Furthermore, as owners are not under constant supervision, they would have the possibility of doing more work earlier on, and some might be more impatient than others starting to do actual work again. However, the owner or caretaker had to record that they performed each step of the exercise schedule according to the protocol. It is also important to acknowledge that mild lameness might not be perceived by the untrained eye.[26] No samples for histology were collected to confirm the ultrasonographic data. As these were client-owned animals, no necropsy or histopathology was performed to assess the presence of abnormal tissue growth following injection. Long-term follow-up was only available for a subset of enrolled horses, as in real-life clinical trial settings, the horses are privately owned and, in the long term, could have been sold, changed careers, changed the location or attended veterinarian and so forth.

To date, most published studies looking into the efficacy of treatments of tendon injuries in horses lack a placebo-controlled design in naturally occurring disease[6,8] or had a limited number of investigated horses.[9,10] Therefore, the current work adds significantly to the level of evidence supporting safety and efficacy of allogeneic stem cells in the treatment of tendon and ligament injuries in horses.

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6 | CONCLUSION

The intralesional treatment with tpMSCs resulted in a significant improvement of swelling, pain to pressure and circumference at the level of injury, lameness, fibre alignment, echogenicity and lesion size, compared to the control group in horses with naturally occurring lesions of the SDFT or SL. Approximately 4 months (112 ± 3 days) following treatment, normalisation of the fibre alignment was achieved in 65.2% of the tpMSCs treated lesions, compared to 9.1% in the control group. Long-term successful outcome was found in the tpMSC treatment group as per significantly lower re-injury rate at 2 years after treatment, compared to control. In conclusion, tpMSCs could provide a promising treatment modality for tendon and ligament lesions in horses and could provide a safe and effective therapeutic for tendon and ligament repair.

AUTHOR CONTRIBUTIONS

Jan H. Spaas, Klaus Hellmann and Gabriele Braun conceived and designed the experiments. Data collection and implementation of the experiments were performed by Stephanie Carlier, Marc Suls, Cedric Bocqué, Justine Thys and Aurélie Vandenberghe. The data was interpreted by Stephanie Carlier, Eva Depuydt, Jan H. Spaas, Klaus Hellmann and Gabriele Braun. The manuscript was drafted by Eva Depuydt and Stephanie Carlier. The manuscript was reviewed by all authors and approved for submission.

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CONFLICT OF INTEREST STATEMENT

Stephanie Carlier, Eva Depuydt, Charlotte Beerts, Klaus Hellmann, Gabriele Braun and Jan H. Spaas were employed or contracted by Boehringer Ingelheim or an affiliated company at the time of the study. The content of this manuscript contains a commercially available stem cell product (RenuTend®) owned and patented by Boehringer Ingelheim. Other authors declare no conflict of interest.

PEER REVIEW

The peer review history for this article is available at https://www.webofscience.com/api/gateway/wos/peer-review/10.1111/evj.14008.

DATA AVAILABILITY STATEMENT

The data that support the findings of this study are available from the corresponding author upon reasonable request.

ETHICAL ANIMAL RESEARCH

The blood collection from the donor horses (EC_2018_002) was approved by an ethics committee, with independent members evaluating the application as approved by the Flemish government (permit number: LA1700607). The study was performed under the clinical trial authorisation number 0004791.

INFORMED CONSENT

A signed owner consent is available for each horse and was signed before inclusion in the study.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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