Treatment of racehorse superficial digital flexor tendonitis – a comparison of stem cell treatments to controlled exercise rehabilitation in 213 cases.

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Key words:

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Background: Overstrain of the superficial digital flexor tendon (SDFT) is a common Thoroughbred (TB) racehorse limb injury requiring treatment.

Objectives: To determine whether treatment of SDFT lesions in flat TB racehorses with autologous bone marrow-derived mesenchymal stem cells (BM-MSCs) or allogenic adipose-derived mesenchymal stem cells (A-MSCs) is associated with improved likelihood of returning to racing, when compared to racehorses managed with a controlled exercise rehabilitation program (CERP) alone.

Study design: Retrospective cohort study combining clinical treatment records with race records.

Methods: Two hundred and thirteen TB racehorses were identified. All were prescribed the same twelve-month CERP. Sixty-six also received intralesional BM-MSC and seventeen A-MSC treatment. Follow-up was a minimum of two years after return to full race training. Multivariable logistic regression models investigated associations

between the treatments and the likelihood of returning to racing and completing five or more (C5+) races post-injury.

Results: Compared to CERP alone, BM-MSC treatment was associated with increased odds of returning to racing (OR 3.19; 95% CI 1.55-6.81) and C5+ races post-injury (OR 2.64; 95% CI 1.32-5.33). Older age and increasing lesion length were associated with a reduced likelihood of returning to racing. Male gender and increased number of pre-injury starts were associated with increased odds of returning to racing. There was no observed increased likelihood of return to racing or C5+ races associated with treatment with A-MSCs compared to CERP alone.

Main limitations: Due to the retrospective nature of the study it was not able to ascertain how strictly the CERP was followed. Due to the novelty of the method, the A-MSC treatment group included a limited number of horses.

Conclusions: In the study population, intralesional BM-MSC treatment was significantly associated with an increased likelihood of returning to racing and C5+ races post-injury compared to CERP alone. Intralesional A-MSC showed no significant association between treatment and the two investigated outcomes.

1. Introduction

Tendon and ligament injuries account for a large proportion of Thoroughbred (TB) racehorse wastage and early retirement. Overstrain injury of the superficial digital flexor tendon (SDFT) constitutes 46% of all racecourse limb injuries and affects 24% of racehorses in training.[1,2] The SDFT operates close to its functional limits in racehorses. Injury is thought to stem from high loads combined with exercise-induced degenerative changes accumulated over time. Overstrain injuries involve fibrillar slippage, breakage of cross-linking, fibrillary rupture and tendon tearing.[3] Due to the poor regenerative capacity of tendon tissue, organised tendon fibril matrix is replaced with scar tissue in the healing process.[4,5] This hypercellular scar tissue is mechanically inferior to normal tendon tissue due to reduced elasticity properties, which contributes to poor functional recovery and a high risk of re-injury.[6,7] Re-injury rates across disciplines range from 16 to 53%, with the highest rates in flat racehorses, followed by National Hunt (NH) racehorses and sports horses.[7,8]

Numerous veterinary regenerative medicine studies have focused on improving tendon tissue's post-injury biomechanical function and reducing re-injury rates. Several equine experimental and clinical reports have supported the use of mesenchymal stem cells (MSCs), also known as mesenchymal stromal cells, for SDFT and suspensory ligament (SL) injuries, with most studies using autologous bone marrow-derived MSCs (BM-MSCs). These report improved tissue architecture, biomechanical function and re-injury resistance.[5,9-11] Studies using a collagenase model of tendon injury in the horse have suggested that BM-MSCs induce a favourable reparative response.[12-13] Reduced re-injury rates have been reported for NH racehorses with naturally occurring SDFT injuries treated with BM-MSCs when compared to historical controls.[14,15] BM-MSCs have also been associated with improved healing in other species.[12,16] Information on the clinical use of adipose-derived MSCs (A-MSCs) in SDFT lesions is limited, but an improved organisation of the extracellular matrix with autologous A-MSCs has been reported.[6]

Usefully, autologous MSCs do not incite an immune response from the host.[17] However, a disadvantage of autologous culture-expanded BM-MSCs is approximately a three-week delay between harvesting and implantation, which allows for fibrosis to begin. Use of allogenic MSCs has the advantage of immediate availability and eliminates factors such as genetic predisposition to injury and natural ageing.[18] Potential

disadvantages of allogenic MSCs are shorter cell survival times following injection and the possibility of initiating an immune-mediated inflammatory response.[19-22]

No previous study has compared racing outcomes of flat racing TB horses that received treatment of SDFT injury with BM-MSCs or A-MSCs compared to a controlled exercise rehabilitation program (CERP) alone. The objective of this study was to determine whether treatment of SDFT lesions in flat racehorses with autologous BM-MSCs or allogenic A-MSCs is associated with an improved likelihood of returning to racing, and C5+ races post-injury, when compared to a control population managed with a CERP alone. We hypothesised that intralesional stem cell treatment (both autologous BM-MSCs and allogenic A-MSCs) combined with a CERP would be associated with an increased likelihood of returning to racing and C5+ races post-injury.

2. Materials and methods

2.1. Study design and data

Horse selection/study population

A convenience sample of client-owned TB racehorses used for flat racing in Australia with a first occurrence unilateral overstrain injury to the SDFT was identified from clinical records at REC Equine Specialists, between 2005 and 2016. These horses had been diagnosed by ultrasonography through REC Equine Specialists and referring veterinarians as having a moderate to severe, acute SDFT core lesion (comparable to *Grade II-IV*)[23]. The paratenon was required to be intact for inclusion in the study. Lesions induced by trauma were not included. The name, age, sex, limb injured, and date of ultrasound examination were gathered from clinical records of REC Equine Specialists. Lesion grade, length, and cross-sectional area (CSA) were also recorded. Lesion length was measured in centimetres (cm) from the point that the core lesion first became visible to the point that it was no longer visible. CSA of the lesion and overall SDFT was measured in cm^2 using the ultrasound machine inbuilt trace function. The CSA of the lesion was calculated as a percentage of the entire SDFT cross-sectional area at

the lesion's most severe (largest) point.

Treated horses

The decision on which horses received autologous BM-MSCs, allogenic A-MSCs, or a CERP alone was made by the horses' owner and trainer, in consultation with the treating veterinarian, as is typical in a commercial private racetrack veterinary clinic.

Bone marrow harvest

Bone marrow was collected in 3ml aliquots from the fourth, fifth and sixth sternebrae under standing sedation and local anaesthesia using an 11G Jamshidi needle (Baxter Healthcare Corporation, McGaw Park, Illinois, USA). A 10MHz linear transducer was used to identify the correct site of the sternum for bone marrow aspiration. Approximately 9ml total bone marrow was aspirated into a 10ml collection syringe, pre-loaded with 0.5ml 5000IU/ml heparin as anti-coagulant. Samples were maintained at 4°C and sent to Vet Biotechnology Ltd (231 Sturt Street, Adelaide, SA, 5000) for cell isolation and expansion for three weeks using a similar procedure as described by Smith *et al.* [24] Stem cells in bone marrow supernatant containing 10 million cells were returned to the veterinarian for implantation in specifically designed transport containers. Lesion injection occurred on the same day the stem cells were received, which was approximately three weeks after bone marrow harvest.

Allogenic stem cells

Allogenic A-MSCs were supplied by Australian Veterinary Stem Cells Pty Ltd (Monash University, Wellington Road, Clayton, VIC, 3800). Stem cells were cultured from cells sourced from selected healthy, young donor animals. Each dose of 2ml contained 21 million stem cells. Lesion injection occurred approximately seven days after ultrasonographic injury diagnosis.

Stem Cell implantation technique

Stem cells were injected into the core lesion of the tendon using ultrasound guidance under standing sedation and local anaesthesia using a technique similar to that outlined by *Smith et al. 2003*. The number of injection sites was not standardised and depended on the extent of lesion and clinician preference. The gauge of needle used varied between 19-21 gauge, depending on clinician preference. After implantation the limb was bandaged immediately to prevent swelling and cutaneous haemorrhage at the injection site. All horses received only one single treatment of stem cells.

Rehabilitation program

All horses, including those that received stem cell therapy and those that were prescribed controlled exercise alone, were given a twelve-month graduated CERP (Table 1). At repeat ultrasound examinations, horses were considered suitable to progress in their rehabilitation if the ultrasonographic appearance of the core lesion had improved, and there was no change, or a decrease in CSA of the SDFT. The exercise program could be shortened or lengthened depending on ultrasonographic healing at three, six, nine and twelve months. It was not possible to determine whether all the ultrasound examinations were performed in each horse or how strictly the recommended exercise program was adhered to.

Racing data

Race records were obtained from Racing Australia (https://racingaustralia.horse/FreeServices/HorseSearch.aspx). Horses were followed up for a minimum of two years after return to full race training (minimum three years total from diagnosis of injury). Whether or not horses returned to racing and whether they completed five or more races post injury was recorded.

2.2. Statistical analysis

This observational study was conducted with an individual horse as the unit of analysis. Separate analyses were performed for two binary outcomes: (i) return to racing after SDFT injury and (ii) completion of five or more

151 races after SDFT injury. Cases were defined as horses that returned to racing – completed at least one race after
152 SDFT injury (outcome/analysis one), and horses that completed five or more races after SDFT injury
153 (outcome/analysis two). Horses that failed to return to racing or failed to complete five or more races post-
154 injury were categorised as controls. Treatment and nine variables identified based on prior studies and a priori
155 hypotheses and specified in Table 2 were investigated for association with the outcomes and to control for their
156 confounding effects. Normality of numerical predictor variables was assessed using the Shapiro-Wilk test of
157 normality. Mean and standard deviation were reported for normally and median and interquartile range for
158 non-normally distributed data. Variables with a limited number of unique values or suspected nonlinear
159 relationship with the outcomes were categorised by terciles or quartiles of the data as indicated in Table 2. The
160 data set included no missing values.
161 Unconditional associations between predictor and outcome variables were assessed by univariable logistic
162 regression models. Variables with a P-value of <0.2 in the univariable analysis were first considered for inclusion
163 in multivariable models. A manual backwards stepwise elimination method was used to construct multivariable
164 logistic regression models. Likelihood ratio tests were used to evaluate nested models. Non-nested models were
165 evaluated using Akaike's Information Criterion. Variables excluded at any stage of the analysis (including the
166 univariable stage) were reinserted into multivariable models and tested for association with the outcome and
167 confounding.[25] The final models' fit was evaluated using the Hosmer-Lemeshow goodness-of-fit test and the le
168 Cessie-van Houwelingen-Copas-Hosmer goodness-of-fit test[26] and by examining the residuals. Statistical
169 significance was set at a P-value of <0.05. P-values were not adjusted for multiple comparisons. All analyses
170 were conducted in R, version 4.1.0.[27]
171
172 **3. Results**
173
174 *Descriptive statistics*
175 The study included 213 flat TB racehorses. The study population involved 60 (28.2%) female and 153 (71.8%)
176 male horses (113 (53.1%) geldings and 40 (18.8%) stallions). The median age at the time of SDFT injury was 4
177 years (interquartile range (IQR) 3 – 4). Left forelimb was affected in 89 (41.8%) horses, right in 124 (58.2%).
178 Grade II lesion was diagnosed in 28 (13.1%), III in 125 (58.7%), IV in 60 (28.2%) horses. The median lesion CSA
179 was 20% (IQR 13 – 30%) and the median lesion length was 10cm (IQR 7 – 14). The median number of pre-injury

race starts in horses included in the study was 7 (IQR 2 – 14) and the median number of pre-injury placements was 3 (IQR 0 – 7). Supplementary Table 1S presents summary statistics for predictor and outcome variables per treatment group; numbers of cases and controls for predictor variables retained in the final models are shown in Table 3.

Of the 213 study horses, 130 (61.0%) were subjected to the CERP alone, while 66 (31.0%) and 17 (8.0%) also received the BM-MSC and A-MSC treatment, respectively. Ninety-five (44.6%) of all 213 horses returned to racing, 57 (26.8%) completed five or more races post-injury. Of the 130 horses subjected to the CERP alone, 51 (39.2%) returned to racing, and 26 (20.0%) completed five or more races. In comparison, of the 66 horses that also received the BM-MSC treatment, 39 (59%) returned to racing, and 26 (39%) completed five or more races, while five of the 17 horses that also received the A-MSC treatment both returned to racing and completed five or more races. Univariable results for both outcomes are presented in supplementary Table 2S.

Return to racing

The final multivariable model for return to racing retained five predictor variables (Table 3). The likelihood of returning to racing was associated with BM-MSC treatment: horses that received BM-MSC treatment in combination with the CERP were more than three times more likely (odds ratio 3.19, 95% confidence interval 1.55 – 6.81) to return to racing than horses subjected to CERP alone. For horses that received A-MSC treatment in combination with the CERP, the odds of returning to racing did not significantly differ from those of horses that were subjected to the CERP alone (0.56, 0.15 – 1.88). A return to racing was also associated with horses' sex: compared to mares, geldings and stallions were more than nine times (9.53, 4.03 – 24.85) and more than two times (2.97, 1.09 – 8.41) more likely to return to racing, respectively. Younger horses were found to have an increased likelihood of returning to racing: compared to four- to seven-year-olds, two- and three-year-old horses were more than four times (4.77, 1.85 – 13.34) more likely to return to racing. Horses with longer lesions were observed to have a lower chance of returning to racing: for each additional centimetre of lesion length, the likelihood of returning to racing decreased by 10% (0.91, 0.85 – 0.97). Finally, horses with three to seven, eight to 14, and 15 to 50 race starts before the SDFT injury were at increased odds of returning to racing compared to horses with zero to two pre-injury starts (4.60, 1.75 – 12.78; 7.57, 2.58 – 24.05; and 3.56, 1.08 –

12.22, respectively). No statistically significant association was observed between returning to racing and lesion CSA or lesion grade at any stage of the analysis.

Completion of five or more races post-injury

The final multivariable model for completion of five or more races post-injury retained three predictor variables (Table 3). The likelihood of C5+ races post-injury was associated with BM-MSC treatment: compared to horses subjected to the CERP alone, horses that received BM-MSC treatment in combination with the CERP were more than twice as likely (2.64, 1.32 – 5.33) to complete five or more races. For horses that received A-MSC treatment in combination with the CERP, the odds of C5+ races post-injury did not significantly differ from those of horses that were subjected to the CERP alone (1.66, 0.46 – 5.39). Completion of five or more races was also associated with horses' sex: compared to mares, geldings were more than two times (2.75, 1.22 – 6.73) more likely to complete five or more races post-injury. For stallions, the odds of C5+ races did not significantly differ from those of mares (1.55, 0.49 – 4.84). Horses with three to 14 race starts before the SDFT injury were at increased odds of C5+ races post-injury compared to horses with zero to two pre-injury starts (3.23, 1.39 – 8.28). For horses that had 15 to 50 race starts before the SDFT injury, the likelihood of C5+ races post-injury did not significantly differ from that of horses with zero to two pre-injury starts (1.78, 0.62 – 5.31). No statistically significant association was observed between C5+ races post-injury and lesion CSA or lesion grade at any analysis stage.

The data contained approximately 10 events per coefficient to support the complexity of the final models.[28] The examination of residuals and the results of the Hosmer-Lemeshow and the le Cessie-van Houwelingen-Copas-Hosmer goodness-of-fit tests, which returned a P-value of ≥0.3, indicated no evidence of a lack of fit for the final models.

4. Discussion

Intralesional treatment with BM-MSCs was associated with more than three times increased likelihood of returning to racing and more than double increased likelihood of C5+ races when compared to CERP alone. C5+ races has previously been suggested to be an appropriate indicator for successful return to productive

performance.[8] In this study, we have used both return to racing and C5+ races as a measure of successful outcome.

We found no increased likelihood of return to racing or C5+ races associated with A-MSC treatment compared to CERP alone. The smaller number of horses, innate and adaptive immune responses to allogeneic cells, and earlier time of treatment could all contribute to reduced effectiveness. Other considerations in the use of allogeneic cells include possible inflammatory responses [19-22] and transmission of viruses .[29] A-MSCs have the ability to differentiate into musculoskeletal tissue but appear to be inferior to BM-MSCs.[30,31] The anatomic location that A-MSCs are collected from is also important, and the ideal site of collection of both A-MSCs and BM-MSCs remains unknown.

There are two studies in mixed breed horses with SDFT and SL injuries treated with allogenic MSCs combined with Platelet Rich Plasma (PRP) showing a beneficial effect, with high numbers of horses returning to their previous level of athletic activity.[32,33] It is unknown how much of the beneficial effects were due to the PRP, which provides a source of growth factors and has been shown to have beneficial effects on tendon healing.[34]

In this study horses were only treated once with stem cells. The majority of literature on this topic describes a single treatment alone, and in the authors experience this is industry standard. However, in two previous studies a second treatment has been administered when follow-up ultrasound examination have shown less than expected results. Beerts *et al*[33] administered a second treatment in 33.3% of SDFT lesions whilst Van Loon *et al*[9] reported a second injection in 4.3% of SDFT lesions. Both studies reported inferior outcomes in horses requiring a second treatment.

There was a difference in dose of cells between each product used in this study; BM-MSCs (10 million cells) and the A-MSCs (21 million cells). These different doses were what was commercially available at the time and both were broadly within a range that would have been expected to have had an effect. Further research and guidelines are required to determine what is the “optimal” dose. Given the large variability in lesion size it is

unlikely that the same dose is suitable for all lesions. There is a possibility that A-MSCs have a different optimal dose than BM-MSCs. Future investigation into the use of A-MSCs is warranted.

Horses with three to 14 race starts before injury were more likely to return to racing and C5+ races compared to horses with zero to two pre-injury starts. Previous evidence on the effect of number of starts prior to injury on likelihood to return to racing is contradictory. Accumulation of tendon injury with an increasing number of starts may increase the risk of future injury,[35,36] but one study has demonstrated that an increasing number of starts is associated with a decrease in risk of tendon injury,[37] presumably due to exercise-induced tissue remodelling rendering the tendons more resistant to injury. Owners of horses with more starts prior to an SDFT injury may potentially be more likely to invest in treatment and rehabilitation compared to owners of unproven horses.

In this study, increasing lesion length was associated with a poorer prognosis. Each additional centimetre in lesion length was associated with a decrease in the likelihood of returning to racing by 10%. However, a statistically significant association was not found between lesion length and the likelihood of C5+ races. No statistically significant association was observed between any of the two outcomes of interest and lesion CSA or lesion grade at either the univariable nor multivariable stage. A previous study described how ultrasound features of SDFT injuries at presentation influence the likelihood of return to racing.[38] They suggested that the CSA of core-lesions was the most significant factor determining successful return to racing with a 29-35% probability of racing again with lesions <50% CSA, decreasing to a probability of 11-16% with lesions >50% CSA. Other studies have demonstrated lesion CSA and length both influence prognosis.[39-41] In this study only six of 213 horses (2.8%) had lesions of >50% CSA, which may account for the lack of association between CSA and return to racing. Other considerations are the variability within and between practitioners when measuring lesion CSA using trace tools on ultrasound machines and the fact that tendon damage is likely not contained to the CSA area measured.

Our study has several important limitations that are inherent to a retrospective study. Firstly, we cannot know how strictly the CERP was followed. Controlled exercise has been shown to have a significant effect on outcome after SDFT injury, with 71% of horses undergoing a controlled exercise program racing at least once, compared to 25% of horses undergoing pasture rest alone.[42] Secondly, variables associated with treatment selection and

treatment protocols were at the discretion of the treating veterinarian in consultation with owners/trainers, which could have affected the results of our study. It is possible that horse ability, value or other unknown factors may have affected which horses received stem cell treatment versus CERP alone. It is also possible that horses receiving costly stem cell therapy were managed more carefully with closer adherence to the CERP. Thirdly, randomisation and blinding would be required to fully understand the treatment effect as equine SDF tendinitis is a highly variable condition where many factors influence prognosis. Finally, factors that prevented return to racing such as SDFT re-injury, injury of another kind, breeding value or other economic factors could not be determined in our study.

Further limitations of the study include lack of documentation of allogeneic immune responses in the A-MSC group. If a non-self immune response occurred, it would be expected to destroy implanted MSCs and possibly prevent their beneficial effect. Future study utilising autologous adipose derived MSCs may be warranted. Another limitation is the different treatment time points between the A-MSC and BM-MSC groups. It seems logical that the optimal time for stem cell implantation is after the acute inflammatory phase has subsided, but before fibrosis begins which is likely to be between week one and two post-injury. However, there is currently insufficient evidence to be sure of the optimal time for MSC implantation.

This is the first study in a population of flat racehorses with naturally occurring SDFT lesions to compare the likelihood of returning to racing, and the likelihood of C5+ races, between CERP alone and CERP combined with intralesional MSC treatments. It provides evidence that intralesional treatment with autologous BM-MSCs is associated with an increase in the likelihood of returning to racing and C5+ race starts compared to CERP alone under the care of a single racetrack practice. This study observed no statistically significant association between A-MSC treatment and returning to racing or C5+ races.

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