Equine Veterinary Journal

Equine Veterinary Journal ISSN 0425-1644
DOI: 10.1111/j.2042-3306.2011.00363.x

Implantation of bone marrow-derived mesenchymal stem cells demonstrates improved outcome in horses with overstrain injury of the superficial digital flexor tendon

E. E. GODWIN, N. J. YOUNG, J. DUDHIA, I. C. BEAMISH and R. K. W. SMITH*

Department of Veterinary Clinical Sciences, The Royal Veterinary College, Hatfield, UK.

*Corresponding author email: rksmith@rvc.ac.uk. Present addresses: N.J. Young, Biotherapeutics National Institute for Biological Standards and Control, Blanche Lane, South Mimms, Herts EN6 3QG, UK. I.C. Beamish, Baker and McVeigh Equine Hospital, Summerveld, Hillcrest, Durban, Kwa-Zulu Natal, 3650, South Africa.; Received: 21.07.10; Accepted: 13.12.10
[Corrections added after online publication 4 November 2011]

Summary

Reasons for performing study: Mesenchymal stem (progenitor; stromal) cell (MSC) therapy has gained popularity for the treatment of equine tendon injuries but without reports of long-term follow-up.
Objectives: To evaluate the safety and reinjury rate of racehorses after intralesional MSC injection in a large study of naturally occurring superficial digital flexor tendinopathy and to compare these data with those published for other treatments.
Methods: Safety was assessed clinically, ultrasonographically, scintigraphically and histologically in a cohort of treated cases: 141 client-owned treated racehorses followed-up for a minimum of 2 years after return to full work. Reinjury percentages were compared to 2 published studies of other treatments with similar selection criteria and follow-up. The number of race starts, discipline, age, number of MSCs injected and interval between injury and treatment were analysed.
Results: There were no adverse effects of the treatment with no aberrant tissue on histological examination. The reinjury percentage of all racehorses with follow-up (n = 113) undergoing MSC treatment was 27.4%, with the rate for flat (n = 8) and National Hunt (n = 105) racehorses being 50 and 25.7%, respectively. This was significantly less than published for National Hunt racehorses treated in other ways. No relationship between outcome and age, discipline, number of MSCs injected or injury to implantation interval was found.
Conclusions: Whilst recognising the limitations of historical controls, this study has shown that MPC implantation is safe and appears to reduce the reinjury rate after superficial digital flexor tendinopathy, especially in National Hunt racehorses.
Potential relevance: This study has provided evidence for the long-term efficacy of MSC treatment for tendinopathy in racehorses and provides support for translation to human tendon injuries.

Keywords: horse; mesenchymal; stem; progenitor; tendinopathy; superficial digital flexor tendon

Introduction

Overstrain injuries to weightbearing tendons are common in cursorial animals that can run fast for long distances. The horse is particularly prone to overstrain injury of the palmar soft tissue structures of the distal limb due to hyperextension of the metacarpophalangeal joint during weightbearing. Of these structures, the superficial digital flexor tendon suffers the highest frequency of injury (Genovese *et al*. 1990; Goodship *et al*. 1994; Kasashima *et al*. 2004). The strain in this tendon is proportional to the force on the limb and hence increases with speed. The most recent epidemiological data suggest that approximately one-quarter of National Hunt racehorses in training are affected by the disease (Dyson 2004; Avella *et al*. 2009; Ely *et al*. 2009), with individual yards reaching frequencies of 40% or more (Pickersgill 2000; Avella *et al*. 2009). In younger flat racehorses the frequency of injury is less at 11%, but increases with age from 6% in 2-year-olds to 16% in >5-year-olds (Kasashima *et al*. 2004). This age-related incidence, together with experimental studies on the influence of exercise on equine tendon suggests that, while injury appears to be spontaneous, occurring most commonly during high speed exercise, it is preceded by degenerative changes occurring within the extracellular matrix (Goodship *et al*. 1994; Smith *et al*. 2002; Birch *et al*. 2008).

Post injury, the equine digital flexor tendon repairs via a process of fibrosis (Williams *et al*. 1980, 1984a,b; Dowling *et al*. 2000; Dahlgren *et al*. 2005) with the scar tissue formed being functionally deficient compared to normal tendon (Crevier-Denoix *et al*. 1997). Although in many cases the healed tendon as a whole can be stronger in the long term than the original tendon (Crevier-Denoix *et al*. 1997), it tends to be stiffer, which has important consequences for the animal in terms of reduced performance and a substantial risk of reinjury. This remains the case in spite of a multitude of treatments that have been proposed (Davis and Smith 2006). As pain is not usually a feature of this condition in the long term, the primary goal of treatment should be to restore functionality, which has therefore encouraged the development of regenerative strategies to improve the quality of reparative tissue.

The optimal *in vivo* regenerative response can be achieved by the combination of 3 key factors (Butler *et al*. 2008): a cell source capable of the formation of an optimal matrix; a scaffold capable of promoting the survival of implanted cells by mechanical protection and/or nutritional support and an anabolic stimulus usually combining, for musculoskeletal tissues, growth factors and appropriate mechanical load to promote optimal extracellular matrix synthesis and organisation.

During the repair process, there is a large influx of cells into the lesion but those cells actually involved in the synthesis of new tissue are believed to be mostly locally derived cells (Williams *et al*. 1980; Cauvin 2000; Kajikawa *et al*. 2007). Most tissues have a sub- or side-population of precursor cells (tissue-specific progenitor cells) used to replenish cells due to natural turnover and aid in repair post injury (da Silva Meirelles *et al*. 2006). Certainly, multipotency has been shown for cells derived from young tendon (Salingcarnboriboon *et al*. 2003; Bi *et al*. 2007). The exact site for these cells within tendon is not known but they are most likely to reside in the endotenon tissue between the collagen fascicles and adjacent to the vasculature (Cauvin 2000). Mature equine tendon, however, does not appear to possess a substantial subpopulation of cells capable of differentiating into multiple cell lines with similar ability to bone marrow derived cells, other than possibly their own cell type (S. Strassberg, P.D. Clegg and R.K.W. Smith - unpublished data), which may explain why this component of the repair process is limited and hence natural repair inferior to normal tendon. We have therefore hypothesised that the implantation of far greater numbers of autologous mesenchymal stem cells (MSCs), than are present normally within tendon tissue, would have the potential of regenerating or improving the repair of the tendon.

Mesenchymal stem cells (MSCs) have been implanted into surgical defects in tendons in multiple *in vivo* experiments in laboratory animals with mostly positive outcomes. Most of these models used surgically created defects in rabbit or rat tendons and have variously shown some improvement in structure and strength of defects implanted with MSCs in a biodegradable scaffold (collagen gel, Vicryl knitted mesh or fibrin glue) over controls implanted with just the scaffold, as assessed by histology or simple biochemical assays (Young *et al*. 1998; Awad *et al*. 1999, 2003; Juncosa-Melvin *et al*. 2007; Butler *et al*. 2008). In other studies using a rat patellar defect model, MSC implantation has been associated with both greater ultimate tensile stress and improved quality of reparative tissue determined by an increased collagen I/III ratio (Hankemeier *et al*. 2005, 2007). Thus, MSC seeded constructs implanted *in vivo* have shown the ability to integrate into the tissue and induce the synthesis of tissue-specific extracellular matrix.

Because of the apparent beneficial effects of MSC implantation in experimental models of tendon injury, we developed a technique to use MSCs for clinical therapies which has attracted much interest. By far the most frequent clinical use has been in the treatment of overstrain injuries of the palmar metacarpal tendons and ligaments (Smith *et al*. 2003; Smith 2008). Two MSC treatment techniques are currently available clinically for the treatment of tendon and ligament injuries in the horse. One utilises cells derived from fat recovered from the tail-head. These cells are recovered by a digestion process that does not include a culture step, so that a mixture of cells is returned to the veterinarian for implantation. This technique has shown some promise in collagenase-induced experimental tendinopathy in the horse (Nixon *et al*. 2008). However, experimental numbers were small and no clinical data on the treatment of naturally-occurring tendinopathy has been published. The alternative technique utilises cultured bone marrow-derived cells, which have been called mesenchymal stem, stromal or progenitor cells (Smith *et al*. 2003). These cells were chosen for this study as they are the most investigated and characterised post natally derived progenitor cells and they appear to perform superiorly to MSCs recovered from other tissues in terms of their ability to undergo chondrogenesis, osteogenesis and adipogenesis under specific conditions (Im *et al*. 2005; Park *et al*. 2006; Vidal *et al*. 2008; Berg *et al*. 2009; Toupadakis *et al*. 2010). In addition, recent experimental data using a collagenase model in the horse have suggested that these cells induced a favourable reparative response (Schnabel *et al*. 2009; Crovace *et al*. 2010). This paper will describe the technique and outcome for the use of bone marrow-derived MSCs for the treatment of naturally occurring superficial digital flexor tendon overstrain injury and compare the results with 2 published studies on the treatment of the same type of injury and followed-up in the same way (Dyson 2004; O'Meara *et al*. 2010).

Materials and methods

Selection of horses

Horses included in for the study were client-owned Thoroughbred racehorses used either for Flat or National Hunt racing in the UK over a 4 year period (2003–07) that had suffered an overstrain injury to the superficial digital flexor tendon. There was no specification as to whether the injury was unilateral or bilateral but the inclusion criteria were first-time tendon overstrain injuries with a ultrasonographically recognisable hypoechoic lesion occupying >10% of the cross-sectional area (CSA) of the tendon at the maximum injury zone within an intact paratenon. Hence, traumatically-induced lesions were not included. It was recommended that injuries should not be recurrent but it was not possible to be certain of this for all treated horses. There was no control of age, sex or trainer for horses included in the study. Horses were treated by their own veterinarian with autologous MSCs recovered from bone marrow as outlined below. Additionally, horses were followed-up for a minimum of 2 years after a return to full work (3 years in total).

Treatment technique with autologous bone marrow-derived mesenchymal progenitor cells

The technique used was modified slightly from that described by Smith *et al*. (2003).

Bone marrow aspiration: Bone marrow was aspirated by the treating veterinarian from 2 sternebrae (usually the fifth and either the fourth or sixth located using ultrasonography) using a 10 cm 11 gauge Jamshidi needle[1]; 9.5 ml bone marrow was aspirated into 10 ml syringes preloaded with 0.5 ml 5000 iu/ml heparin[2] to give a final concentration of 250 iu/ml. After aspiration, at least 2 additional 3.5 ml samples were also obtained in 5 ml plain syringes and transferred immediately to sodium citrate glass blood tubes. These samples were subsequently used to derive bone marrow supernatant to resuspend the MSCs prior to implantation.

MSC culture: This was carried out similarly to that described by Smith *et al*. (2003). The bone marrow was centrifuged at 1500 ***g*** for 10 min without a density gradient centrifugation medium. The buffy coat was removed and plated out into T25 plastic tissue culture flasks with added Dulbecco's Modified Eagles Medium (DMEM) containing 10% fetal calf serum, 100 units/ml penicillin and 0.1 mg/ml streptomycin, 0.11 mg/ml sodium pyruvate and 1% glutamine. After 24–72 h, the unattached cells were washed off with phosphate buffered saline and fresh media added. Adherent cells were cultured until 85–90% confluent (after approximately 14 days) when they were passaged into T225 flasks. About 10×10^6 cells were available for implantation after a further 7 days. The cells were trypinised, centrifuged and counted. Depending on the dose used, 2, 4 or 10×10^6 cells were resuspended in the citrated bone marrow supernatant (centrifuged on arrival and stored at -20°C until the cells were ready for implantation and filtered through a 0.2 μm filter after thawing) at a concentration of 5×10^6 cells/ml.

Implantation: The cells in bone marrow supernatant were transported back to the referring veterinarian in specially designed transport containers consisting of a freezer pack (at -20°C) inside a polystyrene insulated box with a cool pack (at 8°C) separating the freezer pack from the vials of cells. This had been validated to keep the cells at 4–10°C for up to 72 h with only a 9% drop in cell viability every 24 h (data not shown).

Cells were implanted on the same day that they arrived, with the horse under standing sedation as has been described previously (Smith *et al*. 2003; Smith 2008). Briefly, this involved an initial ultrasonographic examination to identify the echogenicity of the core lesion and its extent in order to optimise needle placement for MSC implantation.

To ensure complete desensitisation of the skin overlying the superficial digital flexor tendon, both the palmar nerves deep to the metacarpal fascia and the subcutaneous nerve supply superficial to the fascia were anaesthetised on either side of the limb subcarpally. The palmar metacarpal region was then prepared aseptically.

Most commonly, two 2 ml syringes were each loaded with 5×10^6 MSCs in 1 ml of bone marrow supernatant in a sterile fashion and the cell suspension injected into the core lesion under ultrasound guidance using a 19–21 gauge 38–50 mm needle. The number of injection sites was not standardised and depended on the spread of the injected solution and the extent and maturity of the lesion as visualised ultrasonographically. In most cases, this was 2–4 injection sites along the length of the lesion.

After implantation, the limb was bandaged immediately to minimise subcutaneous haemorrhage and loss of injected cells from the tendon and a single i.m. injection of penicillin was administered to provide perioperative antibiosis.

Rehabilitation programme: After implantation, a standardised exercise programme was prescribed (Table 1). This consisted of initial rest for 7 days (designed to minimise loss of cells from the tendon and for the cells to engraft within the tendon), followed by a gradual increase in the level of exercise over 48 weeks. Walking exercise was recommended in increasing amounts for the first 12 weeks, followed by trotting up to 32 weeks and then cantering and a return to full work after 48 weeks. Repeat ultrasonographic examinations were recommended at 1, 3, 6, 9 and 12 months post implantation and the exercise programme could be shortened or lengthened depending on the ultrasonographic healing. It was not possible to determine whether these ultrasound examinations were always performed or whether the exercise programme was adhered to. The specific exercise programme after 32 weeks post implantation was usually more determined by the owner/trainer to reflect the normal workload of the horse.

TABLE 1: Controlled exercise programme recommended after stem cell treatment

Level	Weeks after implantation	Duration and nature of exercise
Box rest	Preimplantation	Box rest with 10 min walking in hand; Maintain stable bandaging
	1	Box rest with bandaging
Walk	2–4	10 min walking in hand; maintain stable bandaging
1	5–6	20 min walking in hand; maintain stable bandaging
1	7–8	30 min walking in hand; maintain stable bandaging
		Repeat ultrasound examination
2	9–12	40 min walking and 5 min trotting daily
2	13–16	35 min walking and 10 min trotting daily
2	17–20	30 min walking and 15 min trotting daily
2	21–24	25 min walking and 20 min trotting daily
2	25–28	20 min walking and 25 min trotting daily
2	29–32	15 min walking and 30 min trotting daily
		Repeat ultrasound examination
3	33–36	45 min exercise daily with slow canter up to 1 mile twice daily
3	37–40	45 min exercise daily with slow canter up to 1.5 miles twice daily
3	41–44	45 min exercise daily with one 3 furlong gallop 3 times a week
3	45–48	45 min exercise daily with one 6 furlong gallop 3 times a week
3	49–52	Increase exercise level gradually to full race/competition training
		Repeat ultrasound examination for race/competition clearance

Initial safety trial

Six horses (from a variety of disciplines) with large (>40% CSA at the maximum injury zone) core lesions in their superficial digital flexor tendons were treated with 2×10^6 MSCs in 2 ml supernatant as described above. In addition to ultrasonographic examinations at 1, 3 and 6 months, the treated limbs were evaluated by radiography and gamma scintigraphy to detect bone formation within the tendon at 3 months after treatment.

Histological evaluation of treated tendons

Nine treated superficial digital flexor tendons from 8 horses (2 client-owned horses, not included in the clinical data and 6 experimental horses with naturally-occurring injury) that were subjected to euthanasia 150–365 days after implantation, were analysed histologically. Tendons were fixed in 10% buffered formol saline and mounted in paraffin blocks. Longitudinal sections were stained with haematoxylin and eosin and evaluated microscopically for the presence of nontendon tissue.

Follow-up of clinical cases

The database of treated horses was obtained from the VetCell Company but follow-up of horses was independently analysed from race records for National Hunt and flat racehorses for 2 years after a return to full work (3 years after treatment). Reinjury data were obtained by telephone conversation with the referring veterinarian or trainer and entered into a database.

Outcome was based only on those horses that returned to their former function. Horses were excluded from the final analysis if they changed their discipline immediately after treatment (e.g. to become a broodmare or sports horse) or were lost to follow-up. In addition, horses were excluded who had blatantly not followed the rehabilitation programme, as evidenced by a horse returning to racing <8 months after injury. However, in the full population, only one horse was excluded under this criterion.

Information recorded for each horse included the discipline (National Hunt or Flat), age of horse, interval between injury and implantation, the number of cells implanted, the number of starts, and whether the horse had suffered a reinjury or not.

Reinjury was defined as any horse reinjuring the treated or contralateral limb (analysed separately) at any time within the 3 year follow-up period, including the rehabilitation phase. The follow-up strategy was therefore matched to the same protocols used by Dyson (2004) and O'Meara *et al*. (2010) to allow comparison between the MSC treated population in this study and 2 other separate populations that were treated in a variety of other ways (Dyson 2004 Study 1 - controlled exercise and medical treatment with hyaluronan or polysulphated glycosaminoglycans; O'Meara *et al*. 2010 - intralesional insulin-like growth factor 1 injection, firing or desmotomy of the accessory ligament of the superficial digital flexor tendon).

Statistical analysis

Pearson's Chi-squared test was used to assess differences in reinjury rate in racehorses undergoing MPC treatment compared to racehorses receiving other treatments as documented by Dyson (2004) and O'Meara *et al*. (2010). The same test was used to compare the number of horses returning to racing, as well as the effect of the number of stem cells, the age of the horse, and duration between injury and implantation. P values <0.05 were taken to be significant.

Results

Safety

Initial trial: No worsening of the injury was observed clinically or ultrasonographically with no increase in the CSA of the tendons between preimplantation and up to 3 months post implantation (Fig 1). Core lesions were observed to increase in echogenicity quickly although the longitudinal pattern remained inferior to normal tendon (Fig 2). Radiography and scintigraphy performed 3 months after implantation showed no evidence of bone formation (Fig 3).

Histological evaluation of treated tendons: These showed healing with crimped organised collagen fibres and minimal inflammatory cells (Fig 4). There was no evidence of any abnormal tissue or of neoplastic transformation in any of the tissues examined.

Reinjury rate

The data from 141 racehorses treated by intralesional injection of MSCs with 3-year follow-up were available for analysis. Eighteen were lost to follow-up and 10 were retired from racing and changed their career. Of the remaining 113 horses, 111 (98.2%) returned to racing; 31 horses (27.4% of horses with known follow-up) suffered a reinjury to the treated limb and only 6 horses (5.3% of horses with known follow-up) suffered an injury to the contralateral limb. The reinjury rate was significantly lower than that recorded by Dyson (2004) for conservative/medical management alone ($P = 0.0148$; Fig 5; contralateral limb injury not included). Reinjury in National Hunt horses treated with MSCs was also significantly lower (25.7%; $P = 0.0154$) than that recorded by Dyson (2004) in Study 1 (Fig 5) and also when compared to a larger study of National Hunt racehorses by O'Meara (2010), which included contralateral limb injuries ($P = 0.0094$; Fig 5). However, there was no significant difference between reinjury rate in flat racehorses treated with MSCs compared to those treated in other ways (Fig 5).

The average number of cells injected was 9.2×10^6 for the horses that did not reinjure and 7.6×10^6 for those that did, although this was not statistically significantly different (Fig 6a). There was no significant difference in the age of the horse at the time of treatment between those

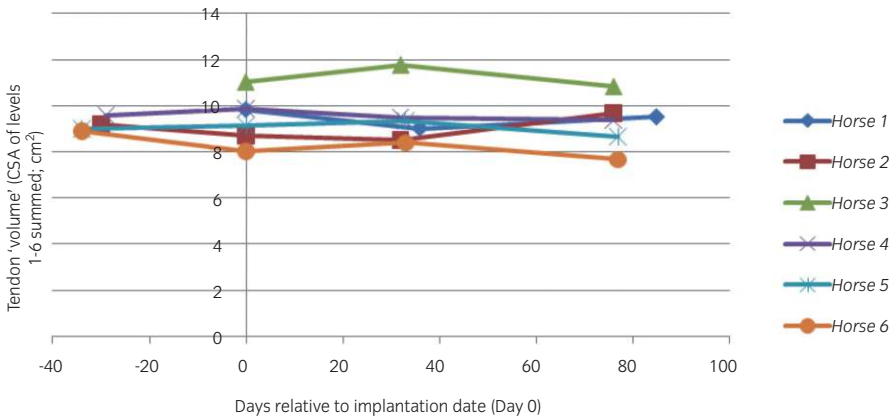

Fig 1: Change in CSA after implantation in the initial safety trial of 6 horses. The average percentage change in tendon 'volume' (calculated from the sum of CSAs of 6 equidistant levels) one and 3 months after implantation was 0.15 and -1.1%, respectively.

horses that did not reinjure (mean age of 9.4 years) and those that did (mean age of 10.5 years). The mean interval between injury and implantation was 46 days for nonreinjuring horses and 53.6 days for reinjuring horses but this was also not statistically different (Figs 6b,c).

Number of starts

Two years after a return to full work, 44.7% of National Hunt racehorses and 25.0% of flat racehorses were still racing. For both National Hunt and flat racehorses, the most common number of race starts was 4–6 (29.1 and 62.5%, respectively, of horses completed these numbers of starts). After receiving MSC treatment, 26.2% of National Hunt racehorses and 25.0% of flat racehorses raced 10 or more times. When compared to those treated by O'Meara *et al*. (2010), the number of horses achieving 3 and 5 starts post treatment was not statistically different (Fig 7).

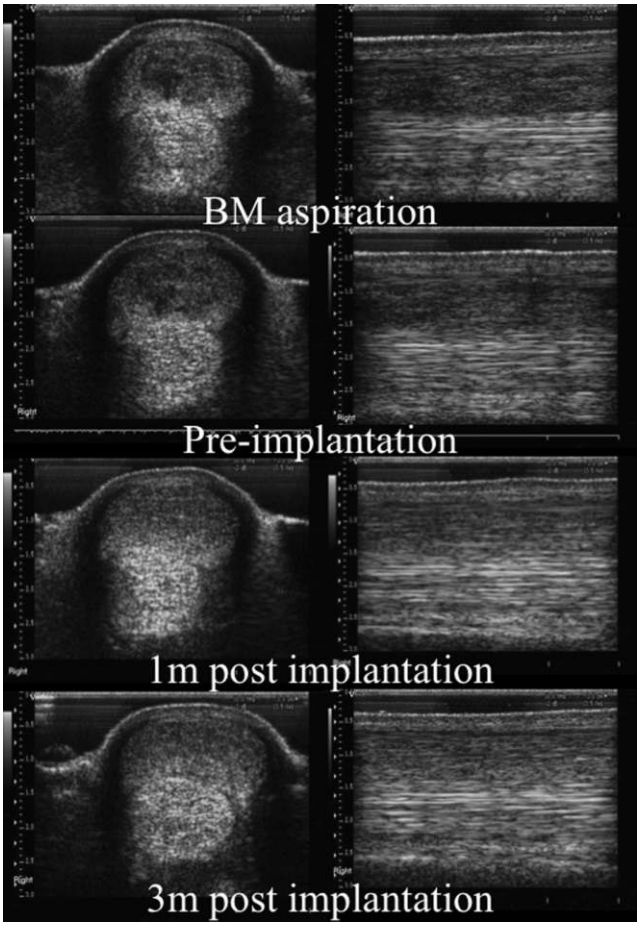

Fig 2: The ultrasonographic appearance of a superficial digital flexor tendon lesion after treatment with 2×10^6 mesenchymal stem cells. Note the rapid filling-in of the lesion and the absence of apparent adverse effects.

Discussion

While comparison of our clinical data with other published series of different treatments does not represent the highest category of clinical evidence, the similarity in selection criteria and analysis between the studies involving UK racehorses provides some confidence that MSC treatment appears to approximately halve the reinjury rate, similar to that reported previously, which was based on a number of nonindependently validated racehorses, including point-to-pointers (Smith 2008).

The 2 racing disciplines were also analysed separately, although the majority of horses in the current study were National Hunt horses. This reflects the higher incidence of superficial digital flexor tendinopathy in this discipline but limits interpretation of the effectiveness of the treatment in the flat racehorse due to low numbers. Power calculations to determine a

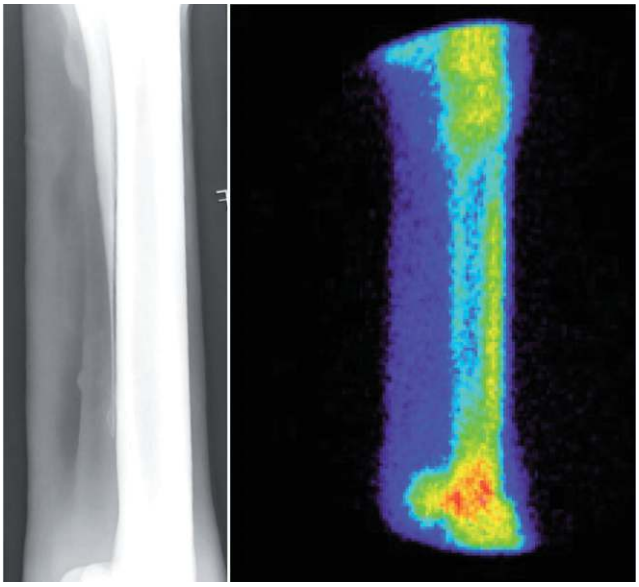

Fig 3: Radiographic (left) and scintigraphic (right) appearance of a treated limb 3 months after implantation. Note the absence of any bone formation in the tendon.

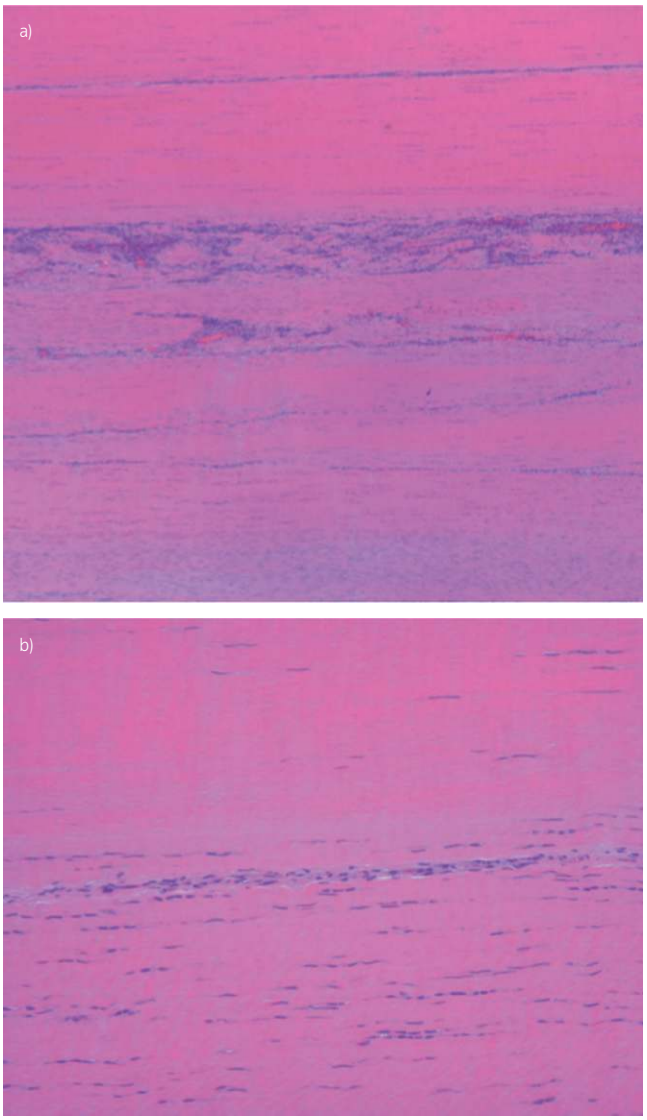

Fig 4: Histological appearance of a superficial digital flexor tendon treated 150 days previously (a) ×20; (b) ×100. Note the well aligned collagen and absence of any aberrant or neoplastic tissue.

significant improvement from 50–25% with 90% power predicts that a group size of 85 horses would be needed to show a significant difference. Therefore, it is not possible to determine the effect of stem cell treatment in flat racehorses because of the small number treated in this study. However, there was still a significant reduction in reinjury rate for this study compared to National Hunt racehorses in Dyson (2004).

The present study demonstrated that a larger percentage of horses treated with MSCs raced at least once compared with other previously published treatments. In a study by Gillis (1997), 71% of horses subjected to a controlled exercise programme raced at least once, while only 25% of horses subjected to pasture rest alone raced again. Other published data show that 20–60% of horses with superficial digital flexor tendinopathy return to racing, although up to 80% of these horses sustain a reinjury (Genovese *et al*. 1990; Marr *et al*. 1993; Fulton *et al*. 1994; Gillis *et al*. 1995; Hawkins and Ross 1995; Hogan and Bramlage 1995; Gibson *et al*. 1997). The number of race starts after treatment did not differ significantly from O'Meara *et al*. (2010), suggesting that MSC treatment did not result in a reduction in post treatment racing frequency compared to intralesional IGF-1, firing or desmotomy of the accessory ligament of the superficial digital flexor tendon. However it was not possible with these data to determine if the racing level had reduced after MSC treatment.

Within populations of naturally occurring injuries there are other variables that could not be quantified, such as the actual rehabilitation exercise programme undertaken, lesion severity and whether the injury was recurrent. While a standardised exercise programme was issued to each horse, the compliance to this programme could not be ascertained and this may have affected the success of the treatment.

Ultrasonographic lesion severity, as determined by lesion CSA and length, has been reported to influence prognosis (Genovese *et al*. 1990, 1997; Marr *et al*. 1993; Reef *et al*. 1997; Reef 2001), although this relationship is not strong (Categories IV–VI had similar failure rates), reflecting both the difficulty in measuring the true hypoechoic area size with indistinct lesions and the fact that the pathology is not restricted to the hypoechoic area seen ultrasonographically (Genovese *et al*. 1997). Unfortunately, lesion size and length data were not available for the cases in this study and so it is not possible to determine if the average lesion size was significantly different from the 2 studies with which the data are compared. However, because treatment with MSCs was only recommended for lesions >10% of the CSA of the tendon and a large group of horses was analysed, we believe it is unlikely that the lesion size differed significantly from the other studies, because the comparator studies had no or a lower minimum size. Furthermore, smaller cohorts of cases from National Hunt horses treated with stem cells were compared to data derived from the same hospital as O'Meara *et al*. (2010) and showed an average lesion CSA of 30–35% for both groups of horses, which was not statistically significantly different from each other (Fig 8).

The MSC treated horses were also found to have statistically significant improved reinjury rates compared to those from the second study reported by Dyson (2004) (treatment with beta-aminoproprionitrile fumarate) when flat and National Hunt horses were combined to give a high enough number (n = 26) and contralateral limb injuries included (P = 0.026). However, the study had specific lesion characteristics for inclusion and hence was deemed to be a less appropriate comparison with the data in the current study.

Recurrent injuries might be considered more likely to reinjure due to pathological changes already present within the tendon, which was the reason for exclusion, although it was not possible to determine this accurately. Of the studies with which these data were compared, one (Dyson 2004) included recurrent injuries (although the percentage of recurrent injuries was not reported), while the other (O'Meara *et al*. 2010), where there was a more highly significant difference, did not.

The number of MSCs injected intralesionally varied within the treated horses for a number of reasons. First, the cell dose was gradually increased

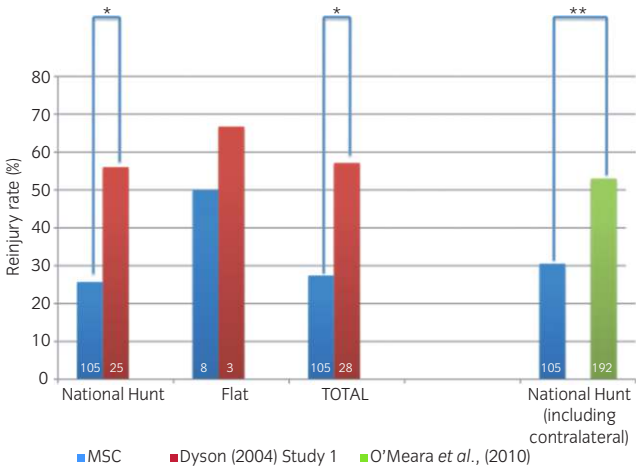

Fig 5: Reinjury rates in racehorses after MSC treatment, analysed for the reinjury of only the treated limb and for both treated and contralateral limbs and compared with published data for other treatments from racehorses selected and followed-up in the same way (Dyson 2004; O'Meara et al. 2010). Case numbers for each group are shown at the base of each column*, ** denotes a statistically significant difference at the P<0.05 and P<0.01 levels, respectively.

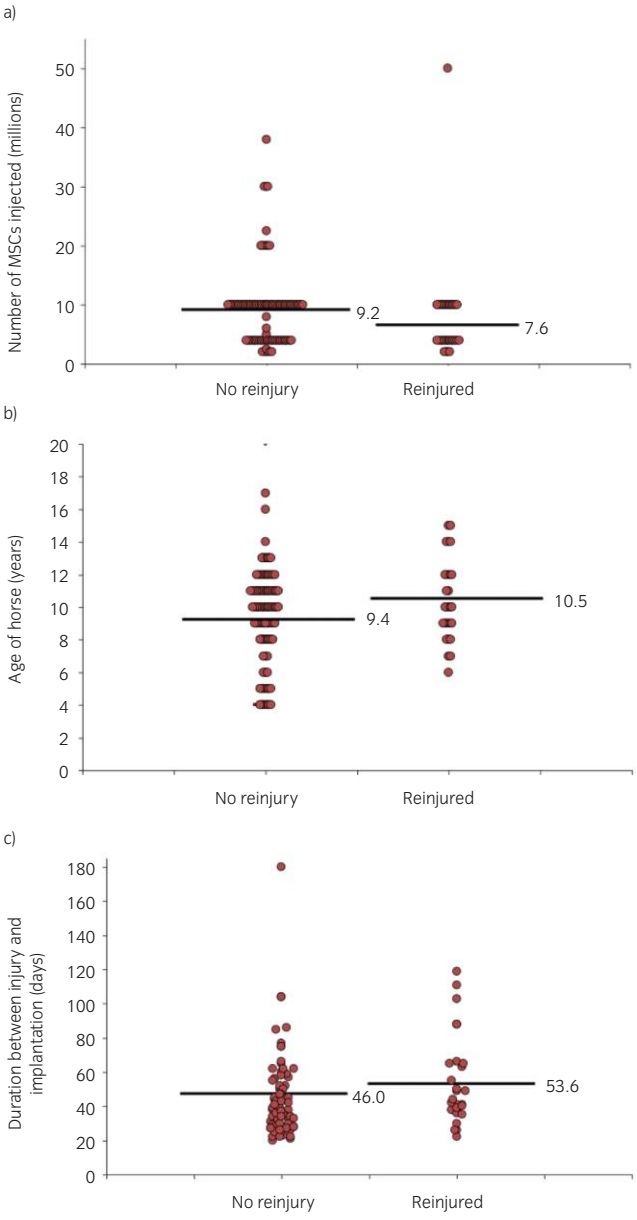

Fig 6: The influence of (a) number of implanted cells, (b) age of the horse and (c) interval between injury and implantation on reinjury. Horizontal bar refers to the mean. There was no statistically significant difference between horses suffering reinjury and those that did not for these parameters.

during the study due to improved culturing techniques, so horses treated earlier generally received fewer MSCs than horses treated later. Second, a higher number of MSCs could be requested for very large lesions. Thus, although there was no significant difference between reinjury rate and number of cells injected, these conflicting factors make interpretation of the effect of cell numbers difficult.

Early analysis of data from a small number of horses suggested a significant difference in the interval between injury and implantation (Smith 2008). However, in this larger, independently validated data series, there did not appear to be a statistically significant effect of interval between injury and implantation although the average interval was longer for the horses reinjuring than those not doing so. When the cases were grouped into ≤5 weeks, 6–8 weeks and >9 weeks to give sufficient group size to calculate a reinjury rate, the reinjury percentage rose from 20.8% for ≤5 weeks, through 24.1% for 6–8 weeks, to 35.0% for ≥9 weeks, although again

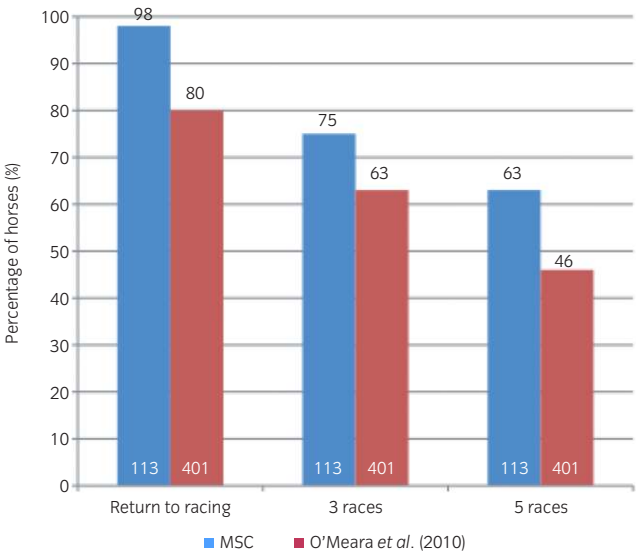

Fig 7: The percentage of MSC treated horses returning to racing and completing 3 and 5 races compared to the data in O'Meara et al. (2010). There were no statistically significant differences between the 2 data sets.

this was not statistically significant because of too few data points. Such an increase in reinjury rate with increasing interval between injury and implantation would be anticipated as horses treated later would have greater amounts of scar tissue within the lesion, making implantation more difficult and potentially reducing the benefits of the MPC therapy.

Although only a cell suspension is implanted into these damaged tendons, the technique still addresses many of the elements required for tendon tissue engineering. The lesion manifests within the central core of the tissue thus providing a natural enclosure for implantation and, by the time of stem cell implantation, is filled with highly vascularised granulation tissue which acts in the role of a scaffold by providing nutritional support of the implanted stem cells. The cytokine and mechanical environment, potentially important drives for differentiation, is provided by the intratendinous location of the cells and the suspension of MSCs in bone marrow supernatant, which has been shown to have significant anabolic effects on cultures of equine tendon and ligament cells (Smith *et al*. 2006; Schnabel *et al*. 2008).

While recently it has been possible to demonstrate that the implanted cells survive in equine tendon (Guest *et al*. 2008, 2010) but in low numbers

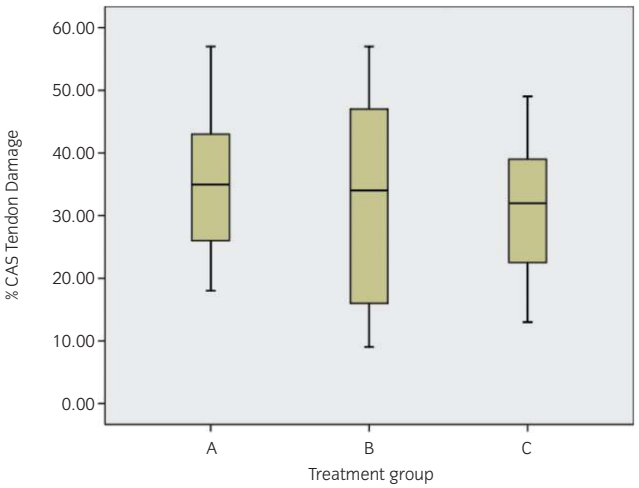

Fig 8: Comparison of lesion size between stem cell treated horses (*Group A*; n = 20), horses treated with IGF-1 (*Group B*; n = 14) and by bar firing (*Group C*; n = 15). There was no significant difference between groups.

(Guest *et al*. 2010), it has not yet been possible to determine the exact role that these implanted cells play in inducing a superior repair that is associated with improved functional recovery, as demonstrated by a reduced reinjury rate. The cells may either differentiate into tenocytes and synthesise the tendon matrix themselves or they may act in a paracrine or trophic fashion to provoke resident cell populations to synthesise new tissue. In addition, MSCs are thought to have profound anti-inflammatory effects via their inhibition of T cell mediated responses (Dazzi and Horwood 2007; Tyndall *et al*. 2007; Karlsson *et al*. 2008; Muller *et al*. 2008). It is not known which of these actions occur after MSC implantation in tendon. Mechanical testing and biochemical and molecular analysis of the new tissue synthesised after treatment will be needed to determine if the resulting tissue demonstrates evidence of regeneration or whether the cells act more to modify the natural repair process to produce better 'quality' tissue.

In most of the horses in the study only one limb was treated because the core lesion was only present on one limb. However, it is known that many of the preceding changes that predispose to clinical injury are bilateral (Smith *et al*. 2002), putting the untreated contralateral limb at risk of subsequent injury when the horse returns to full work. Hence, while subsequent injury to the contralateral limb is not *per se* a failure of the treatment, it is a failure of the case and hence should be considered in follow-up analyses. The data from Study 1 of Dyson (2004) did not include the contralateral limb, while those from O'Meara (2010) did and so both reinjury rates were calculated and compared with the corresponding data from these studies.

There was no evidence of any significant adverse effects after MSC treatment in the horses studied. Evaluation of tendons clinically, ultrasonographically, scintigraphically and histologically showed no evidence of inappropriate tissue or tumour formation. Occasionally needle tracts at the implant site were seen ultrasonographically post treatment but these usually resolved within 3 months after implantation and did not appear to adversely affect the outcome (data not shown).

Thus the results of this study suggest that MSC treatment is more efficacious for the treatment of superficial digital flexor tendinopathy in racehorses compared to other treatments. It is hoped that experience gained from treating naturally-occurring tendon injury in horses will provide sufficient supportive data to encourage the translation of this technology into the human field where large randomised controlled trials will lead to a higher level of clinical evidence.

Authors' declaration of interests

R.K.S. is Technical Adviser for VetCell. Quy Bioscience Ltd., trading as VetCell supplied the data used in this study.

Source of funding

The authors acknowledge the Horserace Betting Levy Board who provided funding for part of this work.

Acknowledgements

The authors would like to thank VetCell, in particular Ms Fiona Cunnington, Mr Greg McGarrell and Mr David Mountford MRCVS, Dr Janet Patterson-Kane MRCVS who performed the histology of the clinical cases, Mr. Bruce Bladon MRCVS for the comparative lesion cross-sectional area analysis and the numerous veterinarians and trainers who contributed information to the database. In addition, they would like to acknowledge the assistance of Dr Tom Witte in the preparation of the manuscript.

Manufacturers' addresses

[1]Cardinal Health, Chateaubriant, France.
[2]CP Pharmaceuticals, Wrexham, UK.

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Author contributions The study was initiated by R.K.W.S., data collection and analysis was by E.E.G. with supporting data provided by N.J.Y., J.D., and I.C.B. R.K.W.S. and E.E.G. equally contributed to the writing of the manuscript.