A new clinical approach: Use of blood-derived stem cells (BDSCs) for superficial digital flexor tendon injuries in horses

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Aims: In this study, we present an innovative therapy using stem cells that were obtained from the peripheral blood of racehorses affected by uninduced superficial digital flexor tendon (SDFT) injuries.

Main methods: Blood-derived stem cells (BDSCs) were generated from the blood samples of three horses in the presence of macrophage colony-stimulating factor (M-CSF). The racehorses received a single autologous BDSC treatment, which resulted in the successful repair of the tendons injuries.

Key findings: The results demonstrated that the BDSCs injection into the damaged tendon stimulated the regeneration of normal tissue. Furthermore, a relationship may exist between the speed and the quality of new tissue formation and the welfare and management of the treated animals.

Significance: This study demonstrates that stem cell technology offers new tools for tissue repair that in many cases is considered incurable, and provides additional evidence that BDScs injections increase the speed and quality of the regeneration process in different animal tissues.

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Introduction

In the veterinary field, the use of stem cells is gaining importance for exploring the potential of regenerative medicine in the treatment of horses with spontaneous tendinitis (Smith et al., 2003; Smith, 2004; Smith and Webbon, 2005; Richardson et al., 2007; Barreira et al., 2008; Fortier and Smith, 2008; Koch et al., 2008). In veterinary use, the most common sources of stem cells are animal adipose tissue or bone marrow. In both cases, the sampling is invasive, and the described protocols fail to provide a homogeneous stem cell population (Cho et al., 2005; Blatt et al., 2005). Our recent in vivo studies have suggested that blood-derived stem cells (BDSCs) can differentiate into a variety of adult cell types, including neurons (Marfe et al., 2011; Spaas et al., 2011). Furthermore, a number of experimental observations have shown that these cells can spontaneously repopulate different organs, such as muscle, bone, liver or heart, and generate differentiated cells in response to acute or chronic damage. These observations, which suggest that these stem cells are capable of contributing to tissue regeneration (Donovan and Gearhart, 2001), have ignited significant interest regarding the ability of cell therapy to repair damaged non-hematopoietic tissues. Large quantities of these cells that are injected into the peripheral blood are likely to lodge within severely damaged organs. The injection of stem cells into the peripheral blood represents a non-invasive and potentially effective approach to easily obtain large quantities of stem cells that are capable of lodging within damaged tissues (Gratwohl et al., 2007). The discovery of the therapeutic potential of stem cells offers new opportunities for the treatment of otherwise incurable diseases.

The digital flexor tendon sheath (DFTS) is a synovial structure that runs from the distal quarter of the metacarpus/metatarsus to the foot on the palmar/plantar aspect of the limb. The sheath contains the superficial and deep flexor tendons, the manica flexoria, digital manica and the palmar/plantar annular ligament, which forms the wall of the sheath as it passes between the proximal sesamoid bones of the metacarpus/tarsalbone angle joint. The manica
flexoria is directly proximal to the palmar annular ligament, is attached to the superficial digital flexor tendon (SDFT), and forms a ring around the deep digital flexor tendon (DDFT) (Dyce et al., 1987). The tendon sheath attaches to the proximal part of the manica flexoria, which forms a blind-ending pouch. Consequently, this pouch has the following 4 potential spaces in the proximal DFTs: the length from palmar/plantar to the SDFT; the space between the SDFT and DDFT; the space between the DDFT and manica flexoria; and the length from the dorsal to the manica flexoria (Dowling et al., 2000; Smith, 2001). These structures can be involved in injuries that would be catastrophic for all types of sport horses. Racing statistics indicate that tendinitis is the most frequent cause of breakdown, and it is often a career-ending event in thoroughbreds (Rossdale et al., 1985; Kasahima et al., 2004). Injuries to the SDFT alone account for an estimated 8% to 30% of all racing injuries (Rooney and Genovese, 1981; Genovese, 1993; Goodship et al., 1994). Moreover, it is typically estimated that the majority of the erythrocytes. The cells were then re-suspended in 5 ml of PBS and incubated for 72 hours at 37 °C in the presence of 50 nM macrophage colony-stimulating factor (M-CSF, Sigma-Aldrich) and 5 μM gentamicin sulfate (Marfe et al., 2011; Spaas et al., 2011).

Flow cytometry analysis

Flow cytometry was used to characterize the stem cells phenotype. The cells were incubated at room temperature for 30 min with anti-human antibodies CD90-PE (phycoerythrin) (BD Biosciences, San Jose, CA, USA), CD117-APC (allophycocyanin) (BD Biosciences, San Jose, CA, USA) or matched isotype controls. Flow cytometry analysis was performed with a BD FACS Aria II cell sorter (BD Biosciences, San Jose, CA, USA).

In situ injection of “deprogrammed” blood-derived stem cells

For treatment, the CD90+ cells were sorted and re-suspended in PBS/gentamicin at a 5-μM concentration. After ultrasonographic localization of the SDFT, the cell suspension was slowly injected in situ using a 2.5 ml sterile syringe. Each animal received only autologous-derived stem cells.

In vivo study

This study was conducted in accordance with ethical procedures and policies approved by the Animal Care Committee (ACC).

Three racehorses, 1 female (aged 20 years) and 2 males (aged 10 and 12 years) with a median age of 12 years (range 10–20) showed a typical core lesion in the BDSC-treated SDFTs upon ultrasonographic examination. A group of three male animals with a median age of 10 years (range 10–15) that were affected by the same disease were also included in this study. This group was treated with conventional therapy.

Results

Isolation of peripheral blood stem cells and flow cytometry analysis

In all of the samples tested, flow cytometry analysis revealed a clear expression of CD90 (PE) and CD117 (APC) on BDSCs that were obtained as described previously (Marfe et al., 2011; Spaas et al., 2011). To isolate the stem cells from peripheral blood, we incubated the nucleated blood cells from each horse with M-CSF for 72 hours as described (Marfe et al., 2011; Spaas et al., 2011). Within this period, the stem cell receptors were expressed on the surface of some cells. Because these cells were quickly dividing after 72 hours, there were a sufficient number of stem cells for sorting. In addition, such sorting resulted in the elimination of differentiated granulocytes and monocytes. Therefore, pure stem cells were obtained. Flow cytometry analysis revealed the clear expression of CD90 (PE) and CD117 (APC) on BDSCs obtained as described in the Materials and methods section after 72 hours in all of the samples tested. This phenotype suggests a commitment toward the hematopoietic stem cell lineage (Fig. 1). Furthermore, a small fraction (approximately 15%),
after sorting, showed the presence of stem cell embryonic markers, such as Sox2, Oct3/4, and Nanog (Table 1).

**In situ injection of deprogrammed blood-derived stem cells**

BDSCs were collected from the same animals as described in the Materials and methods section, and, after the isolation of the autologous peripheral blood stem cells, these cells were inoculated intravenously and locally into the horses. The ultrasonographic examination was performed before and after treatment for each case (Fig. 2).

**Case 1.** A 20-year-old female jumping-horse had an injury to the superficial flexor tendon. The injury at some points of the cross-sectional area reached 80% of the tissue. This horse had been treated with conventional therapy, but as shown by ultrasonographic examination, the disease had not regressed (Fig. 2a). Therefore, the horse was treated with an administration of BDSCs. Four months after the stem cell injection, the tendons appeared to be almost entirely repaired (over 90% filling), and the orientation of the fibers was perfectly parallel to the long axis of the tendon itself (Fig. 2b).

**Case 2.** A 10-years-old, gelding (that was used for dressage) had severe superficial digital flexor tendonitis. The lesion affected 80% of its tendon; the second horse had been treated with conventional therapy, and in this case, there was no regression of the lesion (Fig. 2c). In addition, the horse was treated with the administration of BDSCs. After four months, the tendons appeared to be almost entirely repaired (over 97% filling); the fibers were correctly oriented, and parallel to the long axis of the tendon itself (Fig. 2d).

**Case 3.** A 12-year-old Italian gelding, relapsed with lesions in the right superficial flexor tendon during jumping activities. The lesions affected 30% of the tendon. This case was treated with vesicant therapy without any improvement (Fig. 2e). For this reason, the horse was treated with local and systemic stem cell therapy as described above, and after five months, it was possible to observe the repair of the tendon with an almost 100% reduction of the lesion (Fig. 2d).

In addition, we observed a filling of the lesion and a correct pattern of fibers orientation, which suggested the continuance of a good healing process over this period. In all cases, we observed a reduction in the severity and size of the damaged area, and after three years, no recurrence was found in the treated horses that are presently still active.

On the contrary, the three other horses affected by the same disease and treated with conventional therapy alone showed fibrotic tendon images (Fig. 3a,c,e) and were re-injured within 3 to 6 months of recovery (Fig. 3b,d,f).

**Discussion**

Stem cells represent a promising treatment for certain degenerative or traumatic diseases because of their plasticity and differentiation capacity. Their use in equine veterinary medicine has been intensively studied in recent years, and their regenerative effect, mainly in light of tendon and ligament injuries, has been described in separate in vivo studies (Crovace et al., 2010). In fact, exercise-induced degeneration occurs in equine superficial digital flexor tendons. Increased stiffness, reduced collagen fiber diameter, altered fiber morphology, noncollagenous matrix composition and progressive central hypocellularity have been reported (Patterson-Kane and Firth, 2009). These changes are expected to contribute to tendonitis in athletic horses (Patterson-Kane and Firth, 2009) and are associated with a failure to return to the previous performance level and have a high incidence of recurrence (Smith, 2001). Racing is stressful to the SDFT, and the return to competitions without relapses appears to be a significant measure of functional recovery (Dyson, 2004). Many treatments have been used to facilitate the healing of these lesions, but currently, no treatment has consistently enhanced healing. The methods employed include prolonged periods of inactivity, controlled exercise programs, anti-inflammatory therapy, intra-lesion injections, and peritendinous injection of counterirritants, sclerosing agents, tendon splitting, annular ligament desmotomy, and superior check ligament desmotomy (McIlwraith, 2002).

In the present report, we have described the results obtained in three cases affected by SDFT and treated with the administration of autologous-derived BDSCs. After expansion and sorting, these stem cells were classified into three subpopulations: the first subpopulation was positive for two typical MSC markers, CD90 and CD105, but negative for CD73 (i.e., another MSC marker), which indicated a
commitment of the peripheral blood stem cells towards the MSC lineage; the second subpopulation was positive for CD90, CD117, and CD34, which are typical HSC markers; and the third subpopulation was positive for CD90 and CD117 as well as for Sox2, Oct3/4, and Nanog, which are pluripotent stem cell markers.

Here, we describe the clinical characteristics of the animals with SDFT. In the first case, different therapies were attempted without any success, and therefore, peripheral blood stem cells were used as the last treatment option. The second case was also treated with intravenous stem cell therapy. After four months, we observed a reduction in the severity and extent of the damaged area (over 90% for both animals). The third case, treated with conventional therapy, failed to show any improvement; the damaged lesioned area disappeared within five months but only after BDSC therapy.

In this study, we present a new clinical approach based on the isolation of blood-derived stem cells that were grown in vitro and then injected into the damaged tendon of the same donor horse. With this treatment, we can provide a non-invasive source of stem cells with potentially superior cellular characteristics compared with other stem cells used in veterinary medicine with respect to immune tolerance, proliferative potential and differentiation potency. As far as we know, the use of BDSCs for tendon repair has not been previously described, and extensive reports about their clinical applications in racehorses are lacking. Furthermore, horses treated with conventional methods may recover, but evidence supports a high re-occurrence of tendons injuries (Goodship et al., 1994; Rees et al., 2006). Therefore, all of the conventionally treated horses relapsed shortly after restarting training or competitive activities.

In conclusion, our results show that 30% to 80% of the damaged tendons can recover after a BDSCs injection. In addition, we observed that a greater proportion of normal tendon tissue is produced compared to other therapies (Stashak, 1991; Rees et al., 2006). Our current clinical experience has demonstrated that autologous BDSCs fail to cause any observed significant side effects by either the inoculation process or the formation of different normal tissues within the implantation site. In addition, BDSCs can be a reliable source of stem cells because their use is characterized by the ease of collection and minimal pain and because this process was less invasive compared to other methods described in literature (Wilke et al., 2007; Herthel, 2001; Zuk et al., 2002; Smith et al., 2003; Smith and Webbon, 2005; Strem and Hedrick, 2005; Wilke et al., 2007; Lacitignola et al., 2008; Nixon et al., 2008; Watts et al., 2011; MacLean et al., 2012).

Fig. 2. Horse 1. Ultrasonographic images (7.5 to 12 MHz linear ultrasonographic transducer probe) of the superficial digital flexor tendon of a 20-year-old racehorse with severe superficial digital flexor tendonitis prior to BDSC injection and 4 months after treatment. The lesion affected 80% of the tendon. (a) Transverse and longitudinal images from level 4 (20 cm distal to the 3rd metacarpal bone) before inoculation. (b) Transverse images and longitudinal images (20–24 cm distal to the 3rd metacarpal bone) 4 months after injection. Horse 2. Ultrasonographic images of the superficial digital flexor tendon of a ten-year-old racehorse with severe superficial digital flexor tendonitis prior to BDSC injection and 4 months after treatment. The lesion affected 80% of the tendon. (c) Transverse and longitudinal images from level 4 (20 cm distal to the 3rd metacarpal bone) before inoculation. (d) Transverse and longitudinal images (20–24 cm distal to the 3rd metacarpal bone) 4 months after injection. Horse 3. Ultrasonographic images of the superficial digital flexor tendon of a twelve-year-old racehorse with severe superficial digital flexor tendonitis prior to BDSC injection and 5 months after treatment. The lesion affected 30% of the tendon. (e) Transverse and longitudinal images from level 4 (20 cm distal to the 3rd metacarpal bone) before inoculation. (f) Transverse and longitudinal images (20–24 cm distal to the 3rd metacarpal bone) 5 months after injection.
Furthermore, the in vivo application of stem cells occurred without any side effects in all of the treated cases (Marfe et al., 2011; Spaas et al., 2011). After three years, none of the animals that were successfully treated have shown any side effects, such as rejection, infection or teratoma formation. Therefore, this procedure can significantly improve the healing process of damaged tendons when compared to animals treated with conventional therapies.

Conclusions

The clinical experience that we developed using our protocol has been encouraging in terms of the results, reliability and time of recovery. Of course, more evidence of efficacy, which is essential for full confidence in the technology to be achieved, is highly desirable. It is hoped that experience gained from treating horses with non-induced pathologies will provide sufficient supportive data to promote the application of this technology in humans.

Conflict of interest statement

The authors, M. P. and A.G., declare competing financial interests.

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