Pferdeheilkunde 24 (2008) 4 (Juli/August) 508-512

# Principles of stem cell therapy in the horse – the science behind the technology

*Roger K. W. Smith*

The Royal Veterinary College, North Mymms, U.K.

**Summary**

Tendon and ligament overstrain injuries in the horse are appropriate diseases to consider for stem cell therapy because normal fibrous repair results in reduced functionality, typified by poorer performance and a high rate of re-injury. Exogenously administered mesenchymal stem cells (MSCs) have the potential of inducing a regenerative response because of their capability to differentiate into different cell types. They have been shown to express tendon-related genes when cultured on tendon matrices and are capable of generating 3D structures with many similarities to tendon in vitro. In vivo, implantation of MSCs has demonstrated improved healing of surgically-created tendon defects in laboratory animal models. Based on these observations reviewed here, we have therefore hypothesised that the implantation of autologous mesenchymal stem cells, in far greater numbers than are present normally within tendon tissue, would have the potential of regenerating or improving the repair of the tendon in overstrain injuries in the horse. Our clinical experience of this technology is presented in a separate paper.

**Key words**: Stem cell therapy, orthopedics, technology, science, tendon, tendonitis, horse

**Grundlagen der Stammzelltherapie beim Pferd - die Wissenschaft hinter der Technologie**

Sehnen- und Bandverletzungen des Pferdes eignen sich ganz besonders für den Einsatz der Stammzelltherapie, weil die normale Heilung fibrösen Gewebes in reduzierter Funktionalität resultiert, was sich wiederum in verminderter Leistungsfähigkeit und höherer Rückfallrate ausdrückt. Exogen verabreichte mesenchymale Stammzellen (MSCs) haben aufgrund ihrer Fähigkeit zur Differenzierung in unterschiedliche Zelltypen das Potential, eine regenerative Antwort zu induzieren. Es zeigte sich, dass sie nach Kultivierung auf Sehnenzellmatrix sehnenverwandte Gene exprimieren und dazu fähig sind, in vitro 3 D-Strukturen zu generieren, die viele Ähnlichkeiten mit Sehnen aufweisen. An in Tiermodellen chirurgisch induzierten Sehnendefekten zeigte sich, dass MSCs die Heilung verbesserten. Auf Grundlage der hier beschriebenen Beobachtungen stellen wir deshalb die Hypothese auf, dass die Implantation autologer mesenchymaler Stammzellen in weit größerer Anzahl als sie sich normalerweise in Sehnengewebe finden, das Potential bietet, die Reparaturvorgänge nach Sehnenverletzungen beim Pferd zu verbessern. Unsere klinischen Erfahrungen mit dieser Technologie werden in einem anschließenden Beitrag vorgestellt.

**Schlüsselwörter**: Stammzelltherapie, Orthopädie, Technologie, Wissenschaft, Sehne, Tendinitis, Pferd

## Introduction

Tendon and ligament overstrain injuries still carry relatively poor prognoses in spite of a myriad of treatments that have been tried in the past with few showing any significant improvement in outcome over a prolonged period of rehabilitation consisting of a controlled exercise regime. There has therefore been considerable interest in regenerative strategies for injuries where natural repair mechanisms do not deliver functional recovery. At the forefront of these strategies is the implantation of stem cells where the complex cellular responses after implantation are hoped will mediate an improved therapeutic effect. Stem cell therapy is a fledging technology where many questions still need to be answered but this paper will review the progress in our understanding of the role and effect of stem cell therapy, both in vitro and in vivo in laboratory animal models and in naturally occurring tendon disease in the horse.

## Rationale behind the use of exogenous stem cells to treat tendon over-strain injuries

Tendon naturally heals (repairs) well but the scar tissue formed in this repair is functionally deficient compared to normal tendon, which has important consequences for the animal in terms of reduced performance and a substantial risk of re-injury, in spite of a multitude of treatments that have been proposed. As pain is not usually a feature of these conditions in the long-term, the primary need is to restore functionality and so this has encouraged the development of regenerative strategies.

After injury, there is no deficiency in cell numbers in the tendon. The origin of the invading fibroblasts after injury is not known but possible sources include the resident tendon-derived cells, most likely from a stem cell-like pool within the tendon or surrounding the tendon (eg in the paratenon) – known as intrinsic repair - or from the systemic circulation (most likely derived from the mesenchymal stem cell population within the bone marrow). Recent data from GFP-labelled chimaeric rats subjected to bone marrow transplantation and tendon injury, enable the relative contributions to the repair of bone marrow derived and tendon-derived cells to be evaluated (*Zantop* et al. 2006, *Kajikawa* et al. 2007). This has suggested that the initial cellular infiltrate was systemically derived but likely to be largely white blood cell in nature associated with the inflammatory response and debridement while in the later phases, cells associated with tissue repair, were derived

from the tendon. Given that natural repair produces a functionally inferior structure through the synthesis of scar tissue, a beneficial effect of the addition of an exogenous cell source capable of synthesizing a matrix more resembling tendon tissue can be envisaged. We have therefore hypothesised that the implantation of autologous mesenchymal stem cells, in far greater numbers than are present normally within tendon tissue, would have the potential of regenerating or improving the repair of the tendon.

## Features of equine tendon disease that lends itself to cell therapy

Equine digital flexor tendon strain injuries provide 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 an environment capable of optimizing tendon matrix formation (inside the tendon under tensional load), by the time of stem cell implantation, is filled with granulation tissue which acts in the role of a scaffold. It has the added advantage of being highly vascularised and therefore capable of nutritional support of the implanted stem cells. The cytokine and mechanical environment, which are potentially important drives for differentiation (see below), is provided by the intra-tendinous location of the cells and the suspension of MSCs in bone marrow supernatant which we have shown to have significant anabolic effects on cultures of equine ligament-derived cells (*Smith* et al. 2006).

## The choice of cell source

The potential cell sources for cell-based therapy of tendon and ligament injuries can be divided into differentiated and undifferentiated cells. Every cell source has advantages and disadvantages and these are laid out in Tables 1 and 2.

Currently, autogenous cells are used clinically. An allogenic source would allow an 'off-the shelf' product which could then be given at an optimum time in the disease process rather than one governed by culture times. This would also potentially make the product cheaper. In addition, the product could be standardised more easily although maintaining this standard throughout multiple population doublings necessary to supply sufficient cells would be a concern. MSCs are also not completely immunotolerant although they have been shown to suppress the immune response. Furthermore, allogenic cells would be a possible source of disease transmission and further regulatory issues would need to be addressed.

While the most desirable goal would be to use an optimised specific stem cell line, currently available technology does not enable this to be done. The use of cell surface markers, used to characterise human MSCs, would enable either cell sorting or, at the very least, allow better characterisation of the cultured cell populations which can be highly variable between individuals. However, their use in the horse has been hampered by the lack of specific markers for equine MSCs. Many of the positive stem cell markers described for other species show little of no cross-reactivity (unpublished data). The ability to select tendon tissue promoting MSC populations with or without further modification may be essential in optimising this therapy.

Consequently, the available techniques at this time rely of the use of mixed cell populations. The technique which utilises adipose tissue does not include an enrichment culturing step and so there is a very mixed cell population returned for implantation. Using the bone marrow technique, cell culture is used to enrich the number of stem cells in the cell preparation, although this is unlikely to yield a pure population. It has been recently suggested that the culture step can be avoided for the bone marrow technique, analogous to the fat stem cell technique. The use of the entire nucleated cell population from bone marrow has been assessed in tendon healing models in sheep and horses (*Crovace* and *Lacitgnola* – personal communication). These preparations have a reduced proportion of MSCs but so far the results of these studies have suggested that these preparations may be as effective as the cultured cells. This would have the added advantage of allowing 'horse-side' preparations to be developed that would optimise timing and cost of treatment.

## Demonstrating safety

This is a major concern in the human field because of the concern that stem cells, which, by definition, have self-replicative capacity, may be able to provoke neoplasia when implanted into other tissues. Our current clinical experience in more than 700 horses has demonstrated that implantation of autologous MSCs has not resulted in the formation of dif-

**Table 1** Potential differentiated cell sources / Potential differenzierter Zellquellen

| Cell source | Advantages | Disadvantages |
|---|---|---|
| Tenocytes from flexor tendons | Closest to the cell that synthesises tendon matrix. | Age-related reduction in synthetic ability.<br>Unacceptable donor site morbidity. |
| Tenocytes from extensor tendons | Acceptable morbidity of donor site.<br>No age-related reduction in synthetic ability. | Different phenotypic protein synthesis compared to tenocytes recovered from flexor tendons. |
| Desmocytes from ligament | Higher metabolic activity (therefore potentially more effective matrix synthesis). | Poor donor site morbidity.<br>Different phenotypic protein synthesis compared to tenocytes recovered from flexor tendons. |
| Dermal fibroblasts | Easy to recover.<br>Acceptable donor site morbidity. | Different phenotypic protein synthesis compared to tenocytes recovered from flexor tendons [unpublished data]. |

**Table 2** Potential undifferentiated 'stem cell' sources. The sources in bold are the ones used clinically currently.
*Potential undifferenzierter "Stammzell"quellen. Die fettgedruckten Quellen finden aktuell klinische Anwendung.*

| Cell source | Advantages | Disadvantages |
|---|---|---|
| Embryonic stem cells | True pluripotentiality. | Poorly controllable – teratoma formation when implanted. |
| MSCs from tendon | Evidence of their presence in immature tendon. Endogenous activation possible. | Same as for differentiated cells from tendon.<br>No evidence of their presence in appreciable numbers in adults, necessitating an allogenic source. |
| MSCs from umbilical cord | May have greater multipotentiality.<br>Easy to recover. | Too few numbers in umbilical cord blood.<br>Little data on cells from umbilical cord itself. |
| MSCs from peripheral blood | Easily to recovery. | Present in very small numbers.<br>Requires culture for selection and expansion. |
| **MSCs from bone marrow** | **Easy to recover.**<br>**Extensively researched.** | **More limited differentiation potential.**<br>**Low numbers requiring culture for selection and expansion** |
| **MSCs from fat** | **Easy to recover.** | **More donor site morbidity than for bone marrow.**<br>**More limited knowledge compared to those from bone marrow.** |

ferent normal or abnormal (e.g. tumourous) tissues within the implantation site. One horse has developed ossification in the subcutaneous tissue but not within the tendon itself.

## Evidence of functionality and efficacy – in vitro

The goal in the use of stem cells is to engineer new tendon tissue utilising cellular synthetic machinery. This can be achieved either by the stem cells differentiating into tenocytes and synthesising the tendon matrix themselves or via a paracrine or trophic effect to provoke resident cell populations to synthesise new tissue. It is not known which of these actions occur after stem cell implantation, although current thinking suggests that the latter action may be the most important.

In vitro MSCs cultured in 2D and 3D matrices can be induced to synthesise matrices with some (but not all) the characteristics of tendon extracellular matrix. Equine MSCs can synthesise an abundant and remarkably well structured matrix when cultured in vitro in a bioreactor within the coagulated supernatant of the bone marrow (unpublished data). However, while several confident determinants of multipotentiality (osteogenic, lipidogenic and chondrogenic differentiation) can be shown for bone marrow-derived MSCs, demonstration of tenogenic differentiation has been hampered by the lack of a definitive tenocytic or tendon matrix marker. At present tenocytes are described as having fibroblast morphology (similar to MSCs), and so cannot be identified from appearance alone. Collagen type I is the primary protein synthesized by tenocytes, but this does not differentiate these cells from fibroblasts capable of producing connective tissues, including scar tissue. The synthesis of the glycoprotein Cartilage Oligomeric Matrix Protein (COMP) provides a more discriminating analysis but it too is not specific to tendon although it does have a restricted distribution to tissues primarily designed to withstand load (e.g. cartilage, tendon, and fibrocartilage). The use of a 'signature' of a broad range of synthesised extracellular matrix proteins will enable a better differentiation of most musculoskeletal tissues in the future.
Several key factors can influence the differentiation of MSCs. Implanted cells are influenced by the mechanical environment, contact with resident cells and extraceullar matrix, and soluble growth factors and mediators. Growth and differentiation factors-5, -6 and -7 (GDF-5, -6 and & -7) have been implanted into subcutaneous and intramuscular sites and showed ectopic formation of neotendon/ligament, however the basis of this observation was on type I collagen deposition alone, and therefore might not be conclusive (*Wolfman* et al. 1997). It was hypothesised that MSCs migrated to the area of implantation and differentiated into tendon-like tissue and the exogenous addition of GDF-5 and -6 improved healing of tendon defects. Most recently, the intracellular messenger molecule, Smad 8, has been shown to be associated with tenocytic differentiation (*Hoffman* et al. 2006).

Culturing MSCs on different substrates has been shown to enhance or induce differentiation into different lineages including osteogenic, chondrogenic and neurogenic. MSCs survive well on accellularised tendon matrices and equine MSCs, cultured on fresh acellular equine tendon sections, not only survived, proliferated, and invaded the matrix, but also upregulated COMP gene expression while down-regulating collagen I and III gene expression in comparison to gene expression when cultured on 2D matrices for a short period. Longer contact appears to change the morphology of implanted cells and using this in vitro model, longer culture times (3 weeks) resulted in greater similarities to tenocytes with MSCs lining up with the collagen fascicles.

## Evidence of functionality and efficacy – in vivo

In vivo, MSCs have been implanted into surgical defects in tendons in multiple in vivo experiments in laboratory animals with almost universally positive outcomes *Young* et al. 1998; *Awad* et al. 1999). Most of these models used surgically created defects in rabbit or rat tendons and have variously showed regeneration of new tendon-like tissue in defects implanted with MSCs in a biodegradable scaffold (collagen gel, Vicryl knitted mesh, or fibrin glue) as assessed by histology or simple biochemical assays. However, not all have shown an improvement in microstructure and, because of the use of

allogenic cells, an inflammatory reaction persisted. Furthermore, the implanted cells exhibited fibroblast morphology but were not fully characterised as tenocytes. In more recent studies, MSC implantation was associated with both improved strength and quality of reparative tissue (determined by collagen I/III ratio). Thus, MSC seeded constructs implanted in vivo have shown the ability to integrate into the tissue and synthesise tissue specific ECM, however it is unclear which factors are initiating this functional differentiation.

While it has been possible to demonstrate that the implanted cells survive in equine tendon (*Guest* et al. 2008), it has yet to be shown that once implanted they can synthesise a tendon-like matrix in horse tendon. Mechanical testing and biochemical and molecular analysis of the new tissue synthesised after treatment will be needed to determine if the resulting tissue is of better 'quality' than untreated scar tissue.

Current case follow-up should encouraging signs of efficacy but this data is not able to prove improved healing over conventionally treated animals because no control animals were included. It is frequently not possible to obtain a control population for clinical cases treated in a referral institute within the equine industry. In addition, equine superficial digital flexor tendonitis is a highly variable condition where many factors influence the prognosis. Proof of efficacy will come from controlled experimental studies which are currently being performed and longer follow-up of carefully characterised clinical cases. Recently a surgical model has been developed (*Schramme* – personal communication) that appears more comparable to the natural disease than the previously used collagenase model and that should enable the assessment of the efficacy of autologous stem cell implantation in a controlled fashion.

## Acknowledgements

The author wishes to acknowledge the assistance of Prof. Allen Goodship, Dr. Jay Dudhia, Prof. Gordon Blunn, Dr. Lucy Richardson, Prof. Peter Clegg, and Ms. Stephanie Dakin with the research reported in this paper. Funding for this research was provided by the Horserace Betting Levy Board and Pet-Plan Charitable Trust.

## References

*Awad H. A., Butler D. L., Boivin G. P., Smith F. N., Malaviya P., Huibregtse B.* and *Caplan A. I.* (1999) Autologous mesenchymal stem cell-mediated repair of tendon. Tissue Eng. 5, 267-277

*Guest D. J., Smith M. R.* and *Allen W. R.* (2008) Monitoring the fate of autologous and allogeneic mesenchymal progenitor cells injected into the superficial digital flexor tendon of horses: preliminary study. Equine Vet J. 40, 178-181

*Hoffmann A., Pelled G., Turgeman G., Eberle P., Zilberman Y., Shinar H., Keinan-Adamsky K., Winkel A., Shahab S., Navon G., Gross G.* and *Gazit D.* (2006) Neotendon formation induced by manipulation of the Smad8 signalling pathway in mesenchymal stem cells. J. Clin. Invest. 116, 940-952

*Kajikawa Y., Morihara T., Watanabe N., Sakamoto H., Matsuda K., Kobayashi M., Oshima Y., Yoshida A., Kawata M.* and *Kubo T.* (2007) GFP chimeric models exhibited a biphasic pattern of mesenchymal cell invasion in tendon healing. J Cell Physiol., 210, 684-691

*Smith J. J., Ross M. W.* and *Smith R. K. W.* (2006) Anabolic effects of acellular bone marrow, platelet rich plasma, and serum on equine ligament fibroblasts in vitro. Vet Comp Orthop Traumatol. 19, 43-47.

*Smith R. K., Korda M., Blunn G. W.* and *Goodship A. E.* (2003) Isolation and implantation of autologous equine mesenchymal stem cells from bone marrow into the superficial digital flexor tendon as a potential novel treatment. Equine Vet. J. 35, 99-102

*Wolfman N. M., Hattersley G., Cox K., Celeste A. J., Nelson R., Yamaji N., Dube J. L., DiBlasio-Smith E., Nove J., Song J. J., Wozney J. M.* and *Rosen V.* (1997) Ectopic induction of tendon and ligament in rats by growth and differentiation factors 5, 6, and 7, members of the TGF-beta gene family. J. Clin. Invest. 100, 321-330

*Young R. G., Butler D. L., Weber W., Caplan A. I., Gordon S. L.* and *Fink D. J.* (1998) Use of mesenchymal stem cells in a collagen matrix for Achilles tendon repair. J. Orthop. Res. 16, 406-413

*Zantop T., Gilbert T. W., Yoder M. C.* and *Badylak S. F.* (2006) Extracellular matrix scaffolds are repopulated by bone marrow-derived cells in a mouse model of achilles tendon reconstruction. J. Orthop. Res. 24, 1299-1309

## Reviews

*Richardson L. E., Dudhia J., Clegg P. D.* and *Smith R. K. W.* (2007) Stem cells in veterinary medicine – attempts at regenerating equine tendon after injury. Trends Biotechnol. 9, 409-416

*Smith R. K.* and *Webbon P. M.* (2005) Harnessing the stem cell for the treatment of tendon injuries: heralding a new dawn? Br. J. Sports Med. 39, 582-584

*Taylor S. E., Smith R. K.* and *Clegg P. D.* (2007) Mesenchymal stem cell therapy in equine musculoskeletal disease: scientific fact or clinical fiction? Equine Vet. J. 39, 172-180

*Prof. Roger K. W. Smith MA VetMB PhD DEO DipECVS MRCVS*
*Professor of Equine Orthopaedics*
*The Royal Veterinary College*
*Hawkshead Lane*
*North Mymms, Hatfield, Herts. AL9 7TA*
*U. K.*
*rksmith@rvc.ac.uk*