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Isolation and implantation of autologous equine mesenchymal stem cells from bone marrow into the superficial digital flexor tendon as a potential novel treatment

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Introduction

Superficial digital flexor tendon injuries are a common cause of wastage among competitive horses, associated with failure to return to a previous level of performance and a high incidence of recurrence. Current treatment regimes (reviewed by Dowling *et al.* 2000) have only marginal effects and the major influence on prognosis is the severity of the initial injury. The lysyl oxidase inhibitor, beta-aminoproprionitrile fumarate, was found to have significant improvements in outcome for moderate to severe superficial digital flexor tendonopathy (Genovese 1992), but further clinical trials (Reef *et al.* 1996, 1997) were not as favourable and possible adverse effects of this treatment have been reported (Dahlgren *et al.* 2002). This treatment prevents collagen cross-links forming too early, thereby allowing a controlled exercise regime to improve functionality of scar tissue, but does not regenerate tendon tissue. Scar tissue cannot be as functional as tendon tissue and a goal of future efficacious treatment is therefore to develop methods of regenerating the tissue.

There has been considerable interest recently in the potential therapeutic benefits of mesenchymal stem cells (MSC) for tendon and ligament healing (Woo *et al.* 1999; Caplan and Bruder 2001; Hildebrand *et al.* 2002). These cells reside in small numbers in all tissues and possess multipotential capabilities of differentiating into a number of different tissues. Recent reports have shown that MSCs can be implanted into tendon and ligament tissue in experimental animals (Young *et al.* 1998). One source of the MSCs has been bone marrow, and recently Herthel (2001) reported considerable success in the use of bone marrow aspirated from the sternebrae and injected directly into damaged tendon or ligament. The overall prognosis for a return to full work in 100 horses with suspensory ligament injuries treated with bone marrow was 84%, compared to 15% in a group of 66 horses treated conservatively. However, there is no documentation of the number of fore- and hindlimb injuries, nor the region of the suspensory ligament damaged, all of which are known to have very different prognoses (Dyson 1995, 2000).

Injection of large volumes of bone marrow (30–50 ml) might disrupt remaining intact tendon tissue, including bone spicules and fat cells which can be deleterious to tendon healing, and contain only a small number of MSCs.

This report describes a technique developed for the isolation, characterisation and expansion *in vitro* of equine MSCs, with re-implantation of large numbers of autologous MSCs into a damaged superficial digital flexor tendon in the horse. Whereas MSCs have the potential to differentiate into tenocytes and regenerate tendon matrix after injury, clinical trials using this technique will be necessary to determine whether MSCs are more effective than current treatment methods.

Materials and methods

Case details

Autologous MSCs were re-implanted after expansion *in vitro* into a damaged superficial digital flexor tendon of an 11-year-old polo pony that had suffered a strain-induced injury of the superficial digital flexor tendon 5 weeks previously.

Bone marrow aspiration

After sedation with 10 µg/kg bwt detomidine hydrochloride (Domosedan)[1] and 20 µg/kg bwt butorphanol (Torbugesic)[2], an area 5 x 20 cm over the sternum was prepared by clipping and scrubbing. After aseptic preparation, the intersternebral spaces, easily identified by diagnostic ultrasonography, were marked on the skin using a sterile marker pen. Local anaesthetic solution (2 ml mepivacaine [Intra-Epicaine][3]) was infiltrated subcutaneously over the midpoint in the sagittal plane of 2 adjacent sternebrae. A stab incision with a No. 11 scalpel was made through the skin. A Jamshidi biopsy needle (11 gauge, 10 cm) was introduced approximately 4–6 cm until it contacted the sternebra. It was then pushed a further 3 or 4 cm into the sternebra and 1.8 ml aliquots of bone marrow from each of 2 sternebrae was aspirated into 2 ml syringes, preloaded with 1000 iu heparin (Multiparin[4] 5000 iu/ml). Five aliquots were taken in the first series of aspirates to quantify MSC cell numbers and, thereafter, 2 aliquots were taken for preparation of MSCs for re-implantation. The 1.8 ml marrow aspirates were oscillated gently, transferred into sterile 5 ml tubes and placed on ice for immediate transfer to the laboratory.

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TABLE 1: Quantification of MSCs from sequential bone marrow aliquots after *in vitro* culture

Sample	Cell numbers from colony- forming units (subconfluent) (x 10^5)			Passage 1 (confluence) (x 10^5)		
Horse No.	*1*	*2*	*3**	*1*	*2*	*3**
Days culture	19	16	14	5	5	9
Peripheral blood (control)	-	0	-	-	-	-
Aliquot 1	31 (aliquots 1	9.0	-	78	61.4	-
Aliquot 2	+ 2 combined)	21.4	-		57.8	-
Aliquot 3	-	2.2	-	-	44.6	45.2†
Aliquot 4	7.2 (aliquots 4	16.8	-	44.8	73	-
Aliquot 5	+ 5 combined)	21.8	-		66	-
Average	12.2 (n = 9)			47.1 (n = 10)		

*Horse used for implantation; †sample used for implantation.

Mesenchymal stem cell isolation and in vitro *culture and expansion*

The MSCs were separated using a technique similar to that described for the isolation of MSCs in other species (Rickard *et al.* 1996). In brief, the initial 2 ml of bone marrow aspirate was layered gently onto 4 ml Ficoll (Ficoll Paque PLUS)[5]. This layered mixture was then centrifuged at 1510 rpm (400 ***g***) for 30 mins so that a straw coloured buffy layer formed in between the plasma and Ficoll erythrocyte residue. This buffy layer was recovered and washed by adding 10 ml Dulbecco's Modified Eagles Medium (DMEM[6]; 4500 mg/l glucose, L-glutamine and sodium pyruvate with 10% fetal calf serum, penicillin 50 iu/ml and streptomycin 50 µg/ml). The sample was spun at 2000 rpm (702 ***g***) for 10 mins to remove heparin and Ficoll. The supernatant was discarded and the cell pellet resuspended in 12 ml DMEM. This cell suspension was then added to T75 flasks.

The primary seeded cells were allowed to adhere to the flask for 2 days before changing the medium and, thereafter, the medium was changed every 2 days for 14–16 days when colony-forming units were visible. These cells were passaged before confluency by trypsinisation into T175 flasks and then expanded for a further 5–9 days until confluent.

Cells were removed from the flasks using trypsin digestion and centrifuged at 2000 rpm (702 ***g***) for 10 mins to pellet the cells. The medium supernatant was discarded and the cell pellet resuspended in 1 ml fresh DMEM without serum. A 20 µl aliquot was aspirated and counted in a haemocytometer to give the cell count/ml.

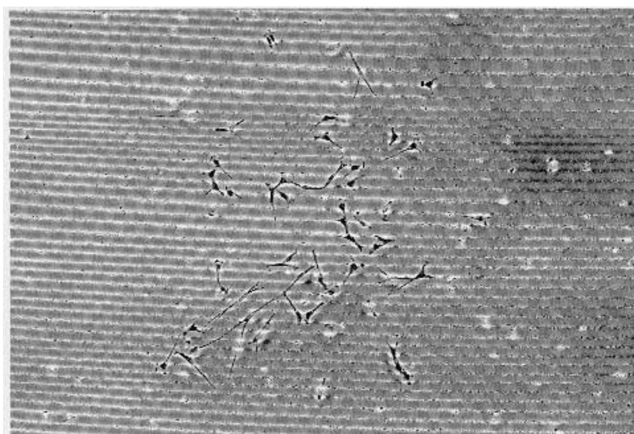

Fig 1: Equine mesenchymal stem cells adhering to plastic after semi-purification from bone marrow by Ficoll centrifugation.

Quantification of MSCs in sequential aliquots of bone marrow

To quantify the yield of MSCs from equine bone marrow, sequential 1.8 ml aliquots of bone marrow from 3 different horses (including the horse in which re-implantation was performed) were cultured separately. Cell numbers were determined after the colony-forming units were established and at confluence after first passage.

Re-implantation of MSCs into superficial digital flexor tendonopathy

Before resuspension in 1.5 ml of plasma, 6.4 x 10^5 cells (to give 40–50,000 cells/0.1 ml injected) were repelletted by centrifugation. The plasma was prepared from freshly obtained blood from the same horse by collecting 10 ml blood into sterile blood tubes containing 500 iu/ml heparin[4] and centrifugation at 1620 ***g*** for 12 mins.

This 1.5 ml cell suspension was then injected into the damaged superficial digital flexor tendon of the same horse from which the cells were originally derived. The injection was performed in an aseptic fashion under sedation and perineural analgesia at the proximal metacarpal site, in 15 x 0.1 ml (approximately 43,000 cells/0.1 ml) injections administered using a 23 gauge, 2.5 cm needle along the length of the lesion from the palmar and medial aspects of the tendon while monitored ultrasonographically. The limb was then bandaged with a standard 3-layered modified Robert Jones bandage.

Results

This protocol resulted in the generation of colony-forming units characteristic of MSCs in other species (Fig 1).

Quantification of MSCs in sequential aliquots of bone marrow

The number of cells recovered before and after passage is shown in Table 1. These cell numbers reflect the relative number of MSCs isolated in aliquots from the same horse as all samples were passaged at the same time. Approximately 10^6 cells were obtained after initial culture for 14–16 days. In addition, it shows that passage expands the cell numbers by a factor of between 2 and 20 times. No MSCs were cultured from a control sample of peripheral blood.

Injury characteristics

There was a central hypoechoic region in the superficial digital flexor tendon which occupied 45% of the cross-sectional area of the tendon at the maximum injury zone and extended from the mid to distal metacarpal region (levels 3–5 [Smith *et al.* 1994]; 16–26 cm distal to the accessory carpal bone). The cross-sectional area of the tendon at the maximum injury zone was 64% larger than the contralateral tendon. The central lesion had already begun to fill with echogenic granulation/fibrous tissue (Fig 2).

Reimplantation of MSCs

Accurate placement of the MSCs into the central tendon lesion was clearly identified ultrasonographically from the air bubbles introduced at the time of injection. The injected cell/plasma mixture was observed to extend proximodistally to the limits of the lesion.

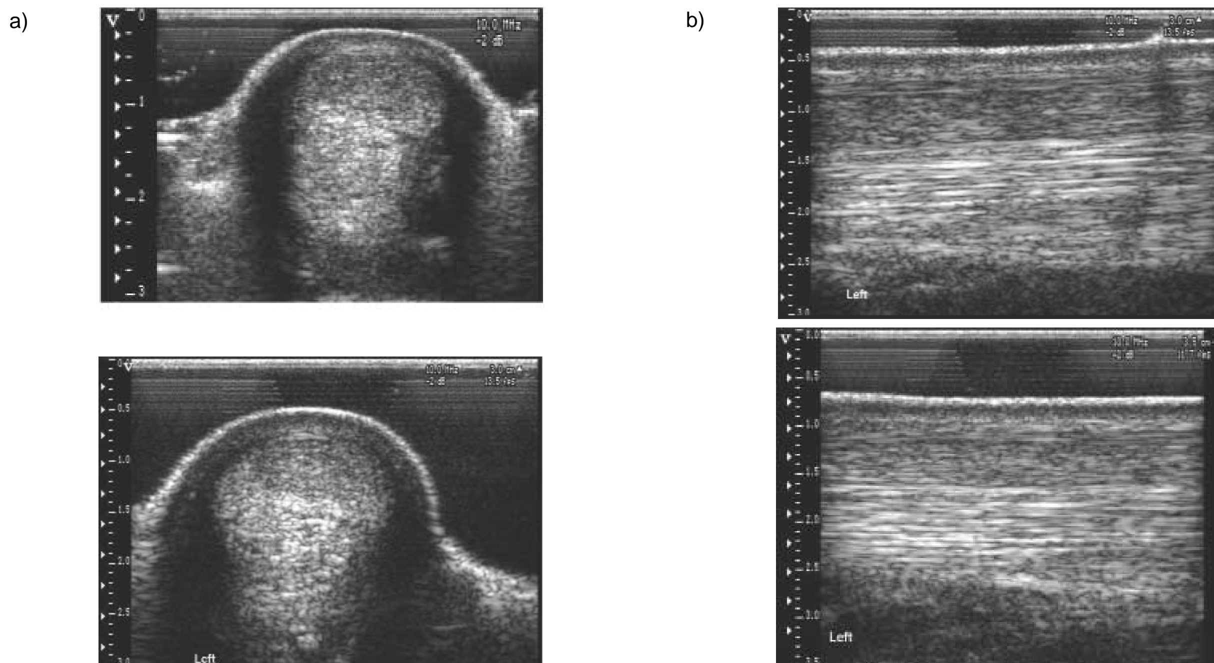

Fig 2: Ultrasonographic images of the superficial digital flexor tendon of an 11-year-old polo pony with a superficial digital flexor tendonitis of 5 weeks duration prior to stem cell implantation and 10 days after stem cell implantation. The lesion occupies the central 45% of the tendon and is filled with granulation/young fibrous tissue. There has been no significant disruption to the healing tendon by the implantation procedure. a) Transverse image from level 4 (20 cm distal to the accessory carpal bone); upper image taken before implantation, lower image taken 10 days after implantation. b) Longitudinal image (20–24 cm distal to the accessory carpal bone); upper image taken before implantation, lower image taken 10 days after implantation.

There was no observable swelling of the limb after the procedure. At re-examination, 10 days and 6 weeks after implantation, there was no lameness at the walk and there was no increased thickening in the region of the superficial digital flexor tendon, although there was mild pain on digital pressure which had disappeared by 6 weeks. Repeat ultrasonography revealed no change in the substance of the tendon (Fig 2). Cross-sectional area measurements from all 7 levels showed minimal change from the pre-implantation (average percentage change for all levels; 0.5% decrease at 10 days, 5% decrease at 6 weeks, with 10% decrease at the maximum injury zone), indicating no disruption to the tendon.

Discussion

This novel technique provides a method for the re-implantation of large numbers of autologous MSCs, which have been expanded in numbers *in vitro*, into the damaged tendon of the same horse. These cells have the potential to produce actual tendon matrix rather than poorly functional scar tissue, as occurs with conventionally managed superficial digital flexor tendonopathy. The signals required to drive MSCs towards differentiation into specific cell types (in this case, tenocytes) are still unclear, although the beneficial use of MSCs in tendon repair has been demonstrated experimentally in rabbits (Young *et al.* 1998). Current studies propose that the introduction of MSCs into the tissue whose cell type is required, together with the mechanical environment experienced by the cell type desired, provides the best stimulus for appropriate differentiation (Carter *et al.* 1998). Equine superficial digital flexor tendonopathy, with its frequent centrally-positioned damage and surrounded either by relatively intact tendon tissue or thick paratenon (which invariably remains intact after even the most severe strain-induced tendon injuries), in a tendon of sufficient size to make accurate intratendinous injection practical, lends itself perfectly as an enclosed vessel in which to implant MSCs. While the implantation of MSCs into other forms of damaged tendons and ligaments (e.g. eccentric lesions) may also prove to be beneficial, accurate placement, retention of cells and minimising iatrogenic trauma caused by the injection process are more problematic.

There was a larger variation in the cell numbers before than after passage because the cell numbers measured before passage were at subconfluence and related to the number of colony-forming units on the plate derived from individual MSCs. Cell numbers at confluence after passage would be expected to be more constant because the cells expand until they cover the whole of the flask surface. Passage is, therefore, often necessary to expand the numbers sufficiently.

An attempt was made to introduce approximately 50,000 cells/0.1ml (approximately 500,000 cells in total). This figure was derived from studies on successful augmentation of fracture healing by MSCs in laboratory animals (R.K.W. Smith, unpublished data). In view of the rapid expansion of cells in vitro after passage, it was expected that this number of cells would be sufficient to populate the central lesion in the tendon. Certainly, *in vitro* expansion of MSCs enables the autologous implantation of considerably larger numbers of MSCs than that available endogenously or delivered by direct injection of bone marrow, and avoids the potential adverse effects of other components of a bone marrow aspirate. In addition, storage of surplus cells frozen provides an additional source of MSCs if required subsequently.

The cells were implanted using blood plasma as a carrier. Platelet-rich plasma, serum, or bone marrow supernatant from the same animal could be used in order to benefit potentially

from a variety of growth factors, although the ideal carrier remains to be determined.

The optimum time for the injection of MSCs is unclear. However, sufficient time had been allowed in this horse for adequate angiogenesis and granulation tissue to form which would be more likely to support MSCs than an earlier haemorrhagic lesion. Abundant growth factors are present in early healing tendon tissue (Cauvin 2001) and the expanded MSCs were therefore delivered into an ideal 'graft bed'.

This preliminary study has demonstrated that the first few millilitres of a bone marrow aspirate from the sternum can yield substantial numbers of MSCs after expansion in culture (in the order of 10^6 cells from 1.8 ml of bone marrow). The technique of equine MSC recovery from bone marrow, *ex vivo* culture and expansion, has been reported previously for experimental animals and horses (Fortier *et al.* 1998), but the re-implantation of these cells into naturally occurring tendonopathy has not been described. The poor success with conventional therapy supports investigation into the use, in equine tendonopathy, of such stem cell therapies, which are generating considerable interest in the human field. The work reported here has demonstrated that the technique is both rational and feasible and paves the way for advances in this field. However, this technique requires further assessment to determine if such MSCs produce tendon, rather than other tissues, especially scar tissue, *in vivo*.

The considerable potential of stem cells recognised in human medicine warrants further investigation in the equine field; and it is hoped that this publication might lay the basis for future research and clinical trials leading ultimately to improvement in the treatment and prognosis of superficial digital flexor tendopathy.

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Manufacturers' addresses

[1]Pfizer Animal Health, Ramsgate, Kent, UK.
[2]Fort Dodge Animal Health, Southampton, Hampshire, UK.
[3]Arnolds, Shrewsbury, Shopshire, UK.
[4]CP Pharmaceuticals, Wrexham, UK.
[5]Amersham Pharmacia Biotech UK Ltd., Little Chalfont, Buckinghamshire, UK.
[6]Sigma Aldrich, Poole, Dorset, UK.

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