

Stem cells in veterinary medicine – attempts at regenerating equine tendon after injury

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Stem cells have evoked considerable excitement in the animal-owning public because of the promise that stem cell technology could deliver tissue regeneration for injuries for which natural repair mechanisms do not deliver functional recovery and for which current therapeutic strategies have minimal effectiveness. This review focuses on the current use of stem cells within veterinary medicine, whose practitioners have used mesenchymal stem cells (MSCs), recovered from either bone marrow or adipose tissue, in clinical cases primarily to treat strain-induced tendon injury in the horse. The background on why this treatment has been advocated, the data supporting its use and the current encouraging outcome from clinical use in horses treated with bone-marrow-derived cells are presented together with the future challenges of stem-cell therapy for the veterinary community.

Current diseases in veterinary medicine for which stem-cell technologies are being considered

Much of the interest in the veterinary field is centred on the use of stem cells for orthopaedic injury and, in terms of translational research for developing the technology for use in the clinic, the most advanced application has been in the horse. Stem cells have been used for studies of heart disease in dogs, although this has been mainly as a model for ischaemic heart disease in man [1]. Unlike humans, most other mammals, including dogs, do not suffer naturally occurring clinical ischaemic heart disease, so the relevance of this research for clinical veterinary medicine is more limited. However, other cardiac diseases, such as canine dilated cardiomyopathy, might be appropriate recipients for this therapy in the future.

Within the equine orthopaedic field, mesenchymal stem cells (MSCs) have been used experimentally and in limited numbers of clinical cases for the surgical treatment of subchondral-bone cysts, bone-fracture repair [2] and cartilage repair [3,4]. However, by far the most frequent use clinically has been in the treatment of overstrain-induced injuries of tendons in horses (Box 1). This review will therefore focus on this disease and on why stem cells have been proposed as a therapeutic option. The techniques available and the current outcome data on their clinical use will be reviewed and the challenges facing the optimisation of this technology discussed.

Rationale behind the use of exogenous stem cells for treating over-strain injuries of the superficial digital flexor tendon

Tendon naturally heals (repairs) well, but the scar tissue formed in this repair is functionally deficient in comparison to normal tendon; this has important consequences for the animal in terms of reduced performance and a substantial risk of re-injury [5]. Because pain is not a feature of this condition in the horse, other than in the initial stages (see Box 2), treatments are aimed at restoring functionality. However, there is little evidence that any of the currently available treatments are more effective than a prolonged period of rehabilitation with carefully controlled exercise [5].

The primary need to restore functionality has encouraged the development of regenerative strategies. However, this goal has been hampered until recently by the inability to define tendon matrix adequately – most analyses reported in the literature cannot differentiate scar tissue from normal tendon tissue. The advent of more comprehensive analyses of tendon extracellular matrix (ECM), including the use of proteomics, is beginning to provide the essential outcome criteria for determining whether tendon has regenerated.

Regenerative medicine requires an exogenous cell source. After injury, tendon does not exhibit a problem with cellular infiltration, but those cells actually involved in the synthesis of new tissue are mostly locally derived cells (see Box 2). Most tissues have a sub- or side-population of precursor cells (tissue-specific progenitor cells) that are used to replenish cells lost because of natural turnover and to aid in post-injury repair. Notable examples are the muscle satellite cells that have been shown to exhibit multipotency, as well as myogenic capacity [6–8]. Multipotent cells from the brain [6], cartilage [8,9], trabecular bone [10], placenta [11], liver, lungs, spleen, thymus [8] and pancreas [8,12] have also been identified. Certainly, evidence of multipotency has been shown for cells derived from tendon [13]. 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 [8]. Although this might be true in young growing tendon, we have not been able to demonstrate that, in adult tendon, there is a cell sub-population that is capable of differentiating into multiple cell lines, other than possibly

their own, with abilities similar to those of bone marrow (BM)-derived cells [14–16]; this might explain why this component of the repair process is limited, and hence, why natural repair is inferior to normal tendon.

Fibroblasts derived from tendon or other sources could be used [17] (Table 1), but the removal of sections of tendon so that cells can be recovered leads to the formation of a secondary lesion at the donor site, and such lesions are unacceptably damaging for flexor tendons in the horse. Furthermore, owing to the different cell characteristics that arise in different tendons and regions of tendon [18], using cells from positional tendons or from other areas of tendon might not be ideal. Alternative cell sources under investigation include dermal fibroblasts, which were shown to be capable of functionally bridging a tendon defect and to have similar histological and tensile properties to the tenocyte-seeded scaffold [19], although *in vitro* these cells behave differently from tenocytes [20].

By contrast, the transplantation of MSCs into a variety of injured skeletal tissues has been shown to promote healing [21–25], and the use of autologous cells has an added benefit because they do not incite an immune response from the host [26]. We have hypothesized that the implantation of autologous MSCs in far greater numbers than are present normally within tendon tissue would have the potential for regenerating or repairing tendon. We envisage that the MSCs could achieve this by one of two roles that can be likened to an orchestra: MSCs act either as the musicians (through differentiation into cells capable of synthesizing tendon matrix) or the conductor (through secretion of factors that induce adjacent cells (implanted or resident) to synthesize tendon matrix) [27,28].

What stem cell sources have been considered for use in horses?

Embryonic stem cells offer great potential because they are pluripotential, but they have the disadvantages of being allogenic (although with greater immunological tolerance) and being associated with a risk of teratoma formation; therefore, these cells are currently not used clinically, although recent work suggests future possibilities [29–31].

MSCs are found in the BM and in small amounts in other tissues, as well as in peripheral blood [32] and the umbilical cord [33,34]. Lee *et al.* [33] have also shown umbilical-cord blood to be a source of MSCs, although Wexler *et al.* [35] deemed umbilical-cord blood and peripheral blood to be an unreliable source. Certainly, work in the horse has not achieved reliable recovery [32]. A potentially better source could be Wharton's jelly in the umbilical cord [34].

Although several cell sources could be considered for the regeneration of tendon in the horse, clinical use has limited the choice to MSCs recovered from BM or fat (see Table 1) because of the ease of recovery, minimal donor-site morbidity and, because these stem cells can be recovered from adult tissue, the possibility of autologous re-implantation, which carries fewer regulatory issues.

MSCs are frequently semi-purified by density centrifugation followed by adhesion to culture plastic,

which, based on findings in other species, is likely to produce an enriched, rather than pure, cell preparation. As with other species, the presence of stem cells within equine MSC preparations has been shown via differentiation of the cells along multiple cell lines through the use of defined media (i.e., usually osteogenic, adipogenic and chondrogenic) [14,36–38].

Can stem cells make tendon? – *in vitro* evidence of tenogenesis

MSCs cultured in 2D and 3D matrices can be induced to synthesize matrices with some (but not all) of the characteristics of tendon ECM. We have found that equine MSCs can synthesize an abundant and remarkably well-structured matrix when cultured *in vitro* in a bioreactor within the coagulated supernatant of the BM (Figure 4). However, although several confident determinants of osteogenic, lipidogenic and chondrogenic differentiation are available, demonstration of tenogenic differentiation has been hampered by the lack of a definitive tenocytic 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 [39], 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 in tissues primarily designed to withstand load (e.g. cartilage, tendon and fibrocartilage). The use of a 'signature' of a broad range of synthesized ECM proteins will enable a better differentiation of most musculoskeletal tissues. In addition, other genes coding for proteins such as scleraxis and tenomodulin show better specificity for tendon, although they are rarely completely specific [40–43].

Several key factors can influence the differentiation of MSCs. Analogous to the planting of seeds, implanted cells are influenced by the mechanical environment ('sun'), contact with resident cells and ECM ('soil') and soluble growth factors and mediators ('water'). Wolfman *et al.* [44] implanted growth and differentiation factors (GDF)-5, -6 and -7 into subcutaneous and intramuscular sites and showed ectopic formation of neotendon; however, the basis of this observation was on type-I-collagen deposition alone, and therefore it might not be conclusive. It was hypothesized that MSCs migrated to the area of implantation and differentiated into tendon-like tissue [44]. Aspenburg and Forslund [45] also showed improved tendon healing when GDF-5 and -6 were implanted into tendon defects, although more recent studies show ectopic cartilage formation associated with GDF-5 [46,47]. Hoffman *et al.* [48] suggested that the expression of the intracellular signalling factor Smad-8 with concurrent stimulation with BMP2 was associated with tenocytic differentiation. Culturing MSCs on different substrates has also been shown to enhance or induce differentiation into different lineages, including osteogenic [49], chondrogenic [50] and neurogenic lineages [51]. MSCs survive well on decellularized tendon matrices [17] and

equine MSCs, cultured on fresh acellular equine tendon sections, not only survived, proliferated and invaded the matrix but also upregulated the COMP gene expression while downregulating collagen I and III gene expression in comparison to gene expression when cultured on 2D matrices [52]. Longer contact appears to change the morphology of implanted cells [53] and, with this *in vitro* model, longer culture times (three weeks) resulted in greater similarities between the way in which tenocytes and MSCs lined up with the collagen fascicles [52].

Can stem cells make tendon? – *in vivo* evidence

Tissue regeneration is thought to require four separate but synergistic elements. There must be a scaffold that will accommodate the cell source to provide protection and nutrition, an appropriate mix of anabolic factors to encourage ECM formation, an appropriate mechanical environment to provide organizational cues and a cell source. Both Cao *et al.* [54] and Juncosa-Melvin *et al.* [55] demonstrated that implanting autologous cells with a scaffold would bridge a tendon defect with better histological characteristics and biomechanical strength than the acellular scaffold alone.

MSCs have been implanted into surgical defects in tendons in multiple *in vivo* experiments with positive outcomes. Young *et al.* [23] used a rabbit model to demonstrate Achilles-tendon-injury repair by MSCs. The defect consisted of a 1 cm excised section in the centre of the tendon, and MSCs were seeded onto a biodegradable scaffold (collagen gel and Vicryl knitted mesh), which was implanted into the defective tendon. The results after 12 weeks showed the regeneration of new tendon-like tissue in the defect. However, an inflammatory reaction persisted, and the new cells exhibited fibroblast morphology but were not fully characterized as tenocytes. Awad *et al.* [24] used a similar rabbit model but investigated injury to the patellar tendon. The results from this study indicated that the MSC-mediated repair improved histological appearance (including more cells and mature collagen fibres), and hence it was postulated that biomechanical properties were improved. However, there was no visible improvement in the microstructure of the tissue compared with the control [24]. More recently, Hankemeier *et al.* [56] demonstrated both improved strength and quality of reparative tissue (by collagen I/collagen III ratio) in a window model in the rat patellar tendon with the use of BM-derived MSCs (BM-MSCs) compared with spontaneous healing, scaffold alone or a differentiated cell control (human ligament cells). The MSC-seeded constructs implanted *in vivo* have shown the ability to integrate into the tissue and synthesize tissue-specific ECM; however, it is unclear which factors are initiating this functional differentiation.

Why the horse? – Disease features that lend themselves to cell therapy

The experimental assessment of tenogenesis by stem cells has utilized laceration injuries in laboratory animals, where maintaining the cells within the laceration site requires some sort of construct, which can also exert an influence, either positively or negatively. By contrast,

equine digital-flexor-tendon strain injuries have a different aetiopathogenesis and provide many of the elements required for tendon-tissue engineering – the lesion manifests within the central core of the tissue and thus provides a natural enclosure for implantation. By the time of stem cell implantation, this enclosure is filled with granulation tissue, which acts in the role of a natural scaffold. It has the added advantage of being highly vascularized and therefore capable of nutritional support of the implanted stem cells. The cytokine and mechanical environment, which are potentially important drives for differentiation (see above), are provided by the intra-tendinous location of the cells and the suspension of MSCs in BM supernatant, which we have shown to have significant anabolic effects on cultures of equine ligament-derived cells [57].

We propose that the optimum time to implant the cells is after the initial inflammatory phase but before fibrous-tissue formation. It was hypothesized that the presence of mature fibrous tissue within the tendon would (i) make implantation more difficult and (ii) reduce the benefits of the stem cell therapy because of its persistence; both hypotheses have been supported by clinical experience of delayed implantation of BM-MSCs, and outcome – successes had an average interval between injury and implantation of 44 days, whereas for horses suffering re-injury, this was 83 days ($p = 0.0035$). Current recommendations are that BM is aspirated within one month of injury and, for the same reason, known recurrent injuries are not considered ideal cases because significant fibrosis would already be present.

Two separate approaches have been used clinically in the horse – one has used a non-adipocyte-cell mixture from fat, and the second has used cultured BM-MSCs. The former does not involve a culture step and therefore has the advantage of cheaper cost and speed of preparation (cells are returned to the practitioner within 48 h). However, the cell mixture is thought to be heterogeneous with regards to cell type. A recent small controlled study using adipose-derived cells in a collagenase model of tendon injury in the horse has indicated some benefit in terms of tissue organization [58]. This technique is widely used only in the USA, although the clinical outcome data for this treatment remain to be reported.

For the BM- MSC technique, BM is recovered from the sternum (or tuber coxae), transferred to a laboratory for culture and expansion of MSCs for three weeks, transferred back to the veterinarian (10×10^6 – 50×10^6 cells, depending on the extent of the lesion) and implanted into the damaged tendon of the same horse under ultrasound guidance [59,60]. This procedure has now become routine in equine clinical practice in the UK, Europe and Australia. So that inappropriate use would be minimized, training courses were run so that veterinarians could learn about the technology, the criteria for treatment and the practicalities of the technique, and more than 400 horses have been treated to date with no adverse effects, other than ultrasonographically evident needle tracts in the tendon after implantation. Current long-term follow-up (>1 year)

of those horses that entered full training (i.e. only those subjected to high-level exercise) showed a re-injury rate of 18%, which compares favourably with a 56% re-injury rate in previous analyses for the same category of horse [61], although this analysis was over two years after a return to full work. Further follow-up of these treated horses after this extended time period will be necessary to allow direct comparison.

Future challenges

Although it has not been possible to demonstrate that the implanted cells survive and synthesize a tendon-like matrix in horse tendon, studies in other species and for other tissues confirm that implanted cells do survive [25,53,62]. Mechanical testing and biochemical and molecular analysis of the new tissue synthesized after treatment will help to determine whether the resulting tissue is of better 'quality' than untreated scar tissue. The use of these markers will enable 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 or no cross-reactivity. The ability to select tendon tissue promoting MSC populations with or without further modification [63] might be essential in optimizing this therapy.

Our current clinical experience has demonstrated that implantation of autologous MSCs does not result in any observed significant deleterious effects, either from the implantation process or from the formation of different normal or abnormal (e.g. tumourous) tissues within the implantation site. We do recognize, however, that these data are 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 [63], and longer follow-up of carefully characterized clinical cases. Recently, a surgical model that appears more comparable to the natural disease than the previously used collagenases model and that should enable the assessment of the efficacy of autologous stem-cell implantation in a controlled fashion has been developed [64].

Conclusions

Our clinical experience has, so far, been encouraging with this technology, although proof of efficacy, essential before full confidence in the technology can be achieved, is still lacking. Although cell-based therapies are likely to be another instrument for tackling orthopaedic disease in the future, it is also likely that we will need to be selective in choosing the right clinical cases. It is hoped that experience gained from treating clinical cases in horses will provide sufficient supportive data to encourage the translation of this technology into the human field.

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Box 1. Horse distal limb anatomy – why are their tendons important?

Tendons join muscle to bone and are usually considered to act by allowing the forces generated by the musculature to act upon the skeleton and initiate movement (locomotion). In large quadrupeds such as the horse, the bulk of the musculature is located proximally to reduce weight in the distal limb, thereby making limb protraction, and therefore locomotion, more efficient. This leads to long tendons, such as the superficial digital-flexor tendon (SDFT), deep digital-flexor tendon (DDFT) and the suspensory ligament (SL) (Figure 1). In addition, the horse has a hyper-extended metacarpophalangeal joint, which means that these long tendons, located on the palmar aspect of the limb, encounter large weight-bearing loads. There are many similarities between the weight-bearing tendons of the horse and those of the human athlete (e.g. Achilles tendon) in their hierarchical structure and matrix composition (Figure 2), their function and the nature of the injuries sustained. In contrast to positional tendons, such as the hand tendons, all weight-bearing tendons also function as springs, absorbing and releasing elastic energy during the different phases of the stride. By doing this, they contribute to the high efficiency of locomotion and also act as a shock absorber for the limb [65]. This is true for both the human and the horse, but the horse has maximized this potential, so that the efficiency of locomotion is in excess of 100% at a gallop [66]. For a spring to function at maximum efficiency, it must be proportionate to the weight applied to it, and it should stretch close to its maximum. Maximal strains in the SDFT of the horse have been recorded at 16% during galloping in thoroughbreds [67]. The strains measured *in vitro*, however, indicate that the failure limit in the SDFT is somewhere between 10% and 20% [68], indicating that the SDFT is operating close to its functional limit.

With a tendon designed to operate close to its functional limit, together with the gradual acquisition of cumulative degenerative damage characteristics in adult horses subjected to high-intensity exercise, it is not surprising that these weight-bearing tendons commonly fail, in both horses and humans. The racehorse is essentially a professional athlete, and therefore it is no great surprise to discover that injuries to the palmarly situated tendons, in particular the SDFT, are very common. All horses used for competitive sport, which includes racing on flat-surfaces or over hurdles (National Hunt), eventing, show-jumping, endurance riding and dressage, can be affected.

Box 2. What happens after injury?

In response to acute injury, there is an initial pronounced inflammatory reaction. Injury-related intratendinous haemorrhage is quickly accompanied by oedema and the infiltration of macrophages, which remove necrotic tissue [68–71]. This inflammatory response is short lived (usually only a few days), and clinically evident inflammation (and pain) is not a feature of chronic injury in the horse. Growth factors and cytokines (PDGF, TGF- β , TGF- α , bFGF and EGF) are released by the invading macrophages and platelets, and these elicit a chemotactic and proliferative response in fibroblasts and encourage the synthesis of collagen types I, III and V, which form scar tissue.

The tendon cellularity is initially increased when compared with normal tendon [70] (Figure 3). The morphology of the invading scar tissue fibroblasts differs from that of the normal tenocytes; they are larger and more basophilic and have large vesicular nuclei, and they are thus more similar to 'myofibroblasts' than tenocytes [71]. The origin of these fibroblasts is not known, but possible sources include the resident tendon-derived cells, probably from a stem-cell-like pool within the tendon or surrounding the tendon (e.g. in the paratenon) – this is known as intrinsic repair – or from the systemic circulation (probably derived from the MSC population within the BM). Recent data from green fluorescent protein (GFP)-labelled chimaeric rats subjected to BM transplantation and tendon injury enable the relative contributions of BM-derived and tendon-derived cells to the repair to be evaluated [72,73]. This has suggested that the initial cellular infiltrate was systemically derived but is likely to be largely white blood cell in nature and associated with the inflammatory response and debridement, whereas, in the later phases, cells associated with tissue repair were derived locally. However, it cannot be ruled out that a small number of systemically derived cells do enter the tendon after injury and exert a paracrine effect on the locally invading cells to initiate a healing response [73].

The newly formed collagen in the scar is less highly cross-linked than that in the normal tendon, and there is much more type III collagen present (<1% type III in normal tendon compared to 20%–30% in tendon scar tissue) [71]. Type III collagen differs from type I because of its smaller fibre diameter, which might provide greater elasticity but confers reduced strength. As the scar increases in size and matures, more-stable cross-links are formed, and the proportion of type I collagen increases, as does fibril diameter. This is associated with an increase in strength and stiffness. The collagen concentration thus returns toward normal; however, the mechanical properties of the tendon are still inferior as a result of a persistently deficient structural organization and composition of the matrix [74]. So that sufficient structural strength can be achieved, larger amounts of fibrous tissue are laid down within and around the tendon, thus giving rise to a tendon that is persistently enlarged but that also has greater structural stiffness. Consequently, this increased stiffness reduces the efficiency of the tendon as a spring and compromises the performance of the horse (or human). Completion of fibrous healing takes a long time (1–2 years), but the tendon is never restored to its previous mechanical properties [68].

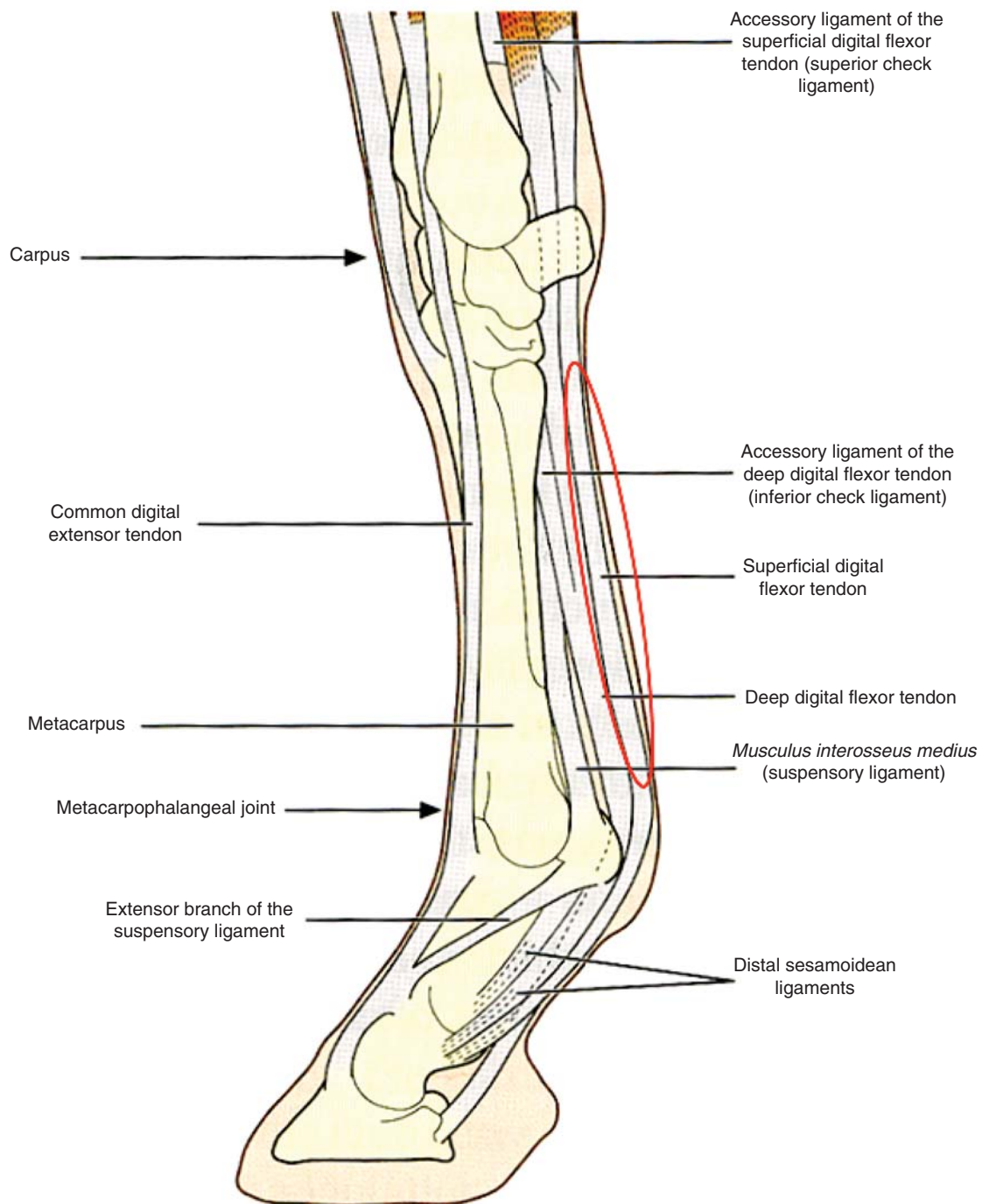


Figure 1. Anatomy of the equine distal forelimb (reproduced, with permission, from Smith and Goodship, 2004 [75]).

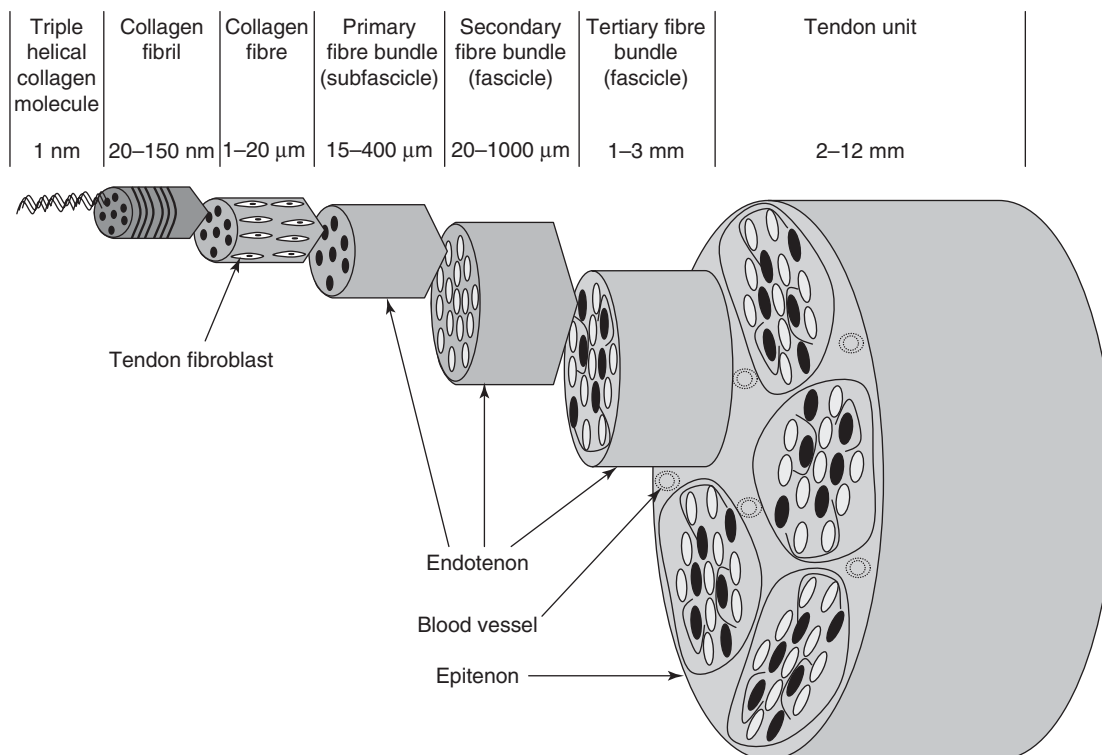


Figure 2. The hierarchical structure of tendon. Tendon tissue has a multi-unit structure composed essentially of the fibril-forming type I collagen molecules, which are organized into individual sub-fascicles and fascicles separated by the ECM of the epitenon. Groups of fascicles form the body of the functional tendon, within which is a rich, vascularized and innervated ECM that is synthesized and maintained by the tendon fibroblasts, which typically align longitudinally in rows along the collagen fibres. The relative scale for the multi-unit components is shown (adapted from Wang, J.H. [74]).

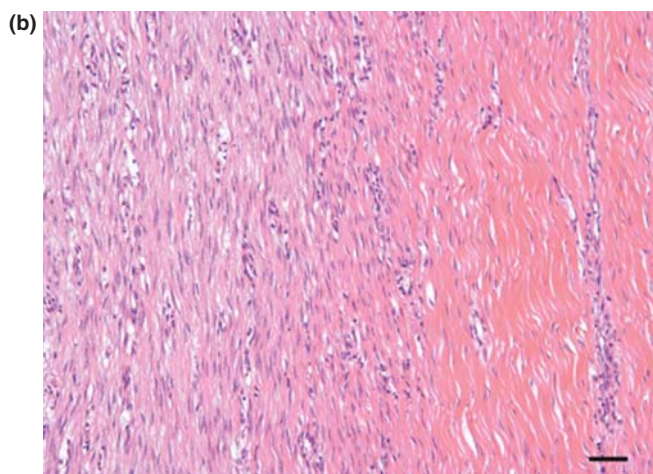
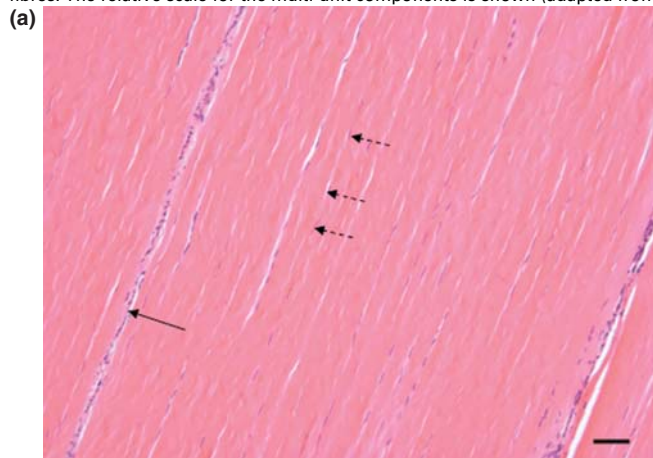


Figure 3. Haematoxylin- and eosin-stained longitudinal sections of healthy young superficial digital-flexor tendon **(a)**, demonstrating the longitudinally aligned tenocytes (dashed arrow) and interfascicular tissue (solid arrow), and scar tissue four months after injury **(b)**, demonstrating the disorganized matrix and the increased cellularity and vascularisation (arrows indicate blood vessels). Scale bar = 100 μ m. (Courtesy of Janet Patterson-Kane).

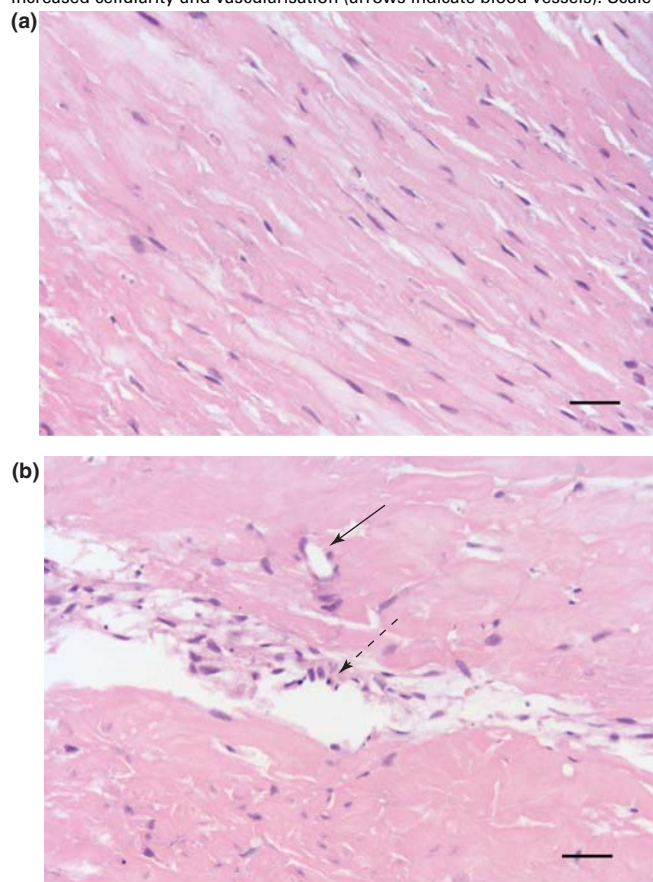


Figure 4. Tenogenesis *in vitro* by BM-MSCs. Equine BM-MSCs were cultured in a bioreactor for three weeks in BM supernatant at a concentration of 5 million cells per ml supernatant. Haematoxylin- and eosin-stained sections show cells that are dispersed from neighbours and predominantly aligned axially; the organization of these cells thus bears a resemblance to the cellular organization of native tendon tissue **(a)**. Open structures (bold arrow) that might be rudimentary blood vessels and hypercellular areas similar to interfascicular tissue (dashed arrows) are also visible **(b)**. Scale bar = 100 μ m. (Courtesy of Janet Patterson-Kane).

Table 1. Possible sources of cells for tendon-tissue engineering

Cell source	Advantages	Disadvantages
Differentiated cells		
Tenocytes from flexor tendons	Closest to the cell that synthesizes appropriate tendon matrix.	Age-related reduction in synthetic ability. Unacceptable donor site morbidity.
Tenocytes from extensor tendons	Acceptable morbidity of donor site. No age-related reduction in synthetic ability.	Different phenotypic protein synthesis than tenocytes recovered from flexor tendons.
Dermal fibroblasts	Easy to recover; acceptable donor site morbidity.	Different phenotypic protein synthesis than tenocytes recovered from flexor tendons.
Undifferentiated cells		
Embryonic stem cells	True pluripotentiality.	Poorly controllable – teratoma formation when implanted.
Mesenchymal stem cells from tendon	Evidence of their presence in immature tendon. Endogenous activation possible.	Same as for differentiated cells from tendon. No evidence of their presence in appreciable numbers in adults, necessitating an allogenic source.
Mesenchymal stem cells from umbilical cord	Might have greater multipotentiality. Easy to recover.	Too few numbers in umbilical-cord blood. Few data on cells from umbilical cord itself.
^a Mesenchymal stem cells from bone marrow	Easy to recover. Extensively researched.	More limited differentiation potential. Low numbers requiring culture for selection and expansion.
^a Mesenchymal stem cells from fat	Easy to recover.	More donor-site morbidity than for bone marrow. Limited knowledge about these cells compared to those from bone marrow.

^aCell sources used for the treatment of tendon over-strain injuries in the horse.