



## SPONTANEOUSLY ARISING DISEASE: REVIEW ARTICLE

# The Pathogenesis of Tendon Microdamage in Athletes: the Horse as a Natural Model for Basic Cellular Research

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## Summary

The equine superficial digital flexor tendon (SDFT) is a frequently injured structure that is functionally and clinically equivalent to the human Achilles tendon (AT). Both act as critical energy-storage systems during high-speed locomotion and can accumulate exercise- and age-related microdamage that predisposes to rupture during normal activity. Significant advances in understanding of the biology and pathology of exercise-induced tendon injury have occurred through comparative studies of equine digital tendons with varying functions and injury susceptibilities. Due to the limitations of in-vivo work, determination of the mechanisms by which tendon cells contribute to and/or actively participate in the pathogenesis of microdamage requires detailed cell culture modelling. The phenotypes induced must ultimately be mapped back to the tendon tissue environment. The biology of tendon cells and their matrix, and the pathological changes occurring in the context of early injury in both horses and people are reviewed, with a particular focus on the use of various tendon cell and tissue culture systems to model these events.

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## Introduction

Tendon injuries associated with athletic activity and ageing are most frequent and important in horses and people. Healing is prolonged, with failure to reconstitute normal structure contributing to high injury recurrence rates in both species (Crevier-Denoix *et al.*, 1997; Ely *et al.*, 2009; Molloy and Wood, 2009). Failure of regeneration suggests that prevention would have a significantly greater clinical impact than therapy. It is generally agreed that tendons sustain cumulative subclinical damage (i.e. microdamage) for undefined periods of time prior to rupture, implying that there is a ‘window of opportunity’ for intervention. Although our understanding is increasing, knowledge is still limited in terms of: exactly how and why certain tendons accumulate cellular and matrix microdamage; the effects of microenvironments experienced by tendon cells during and after exercise on their viability and function including injury detection and repair capacity; and many aspects of fundamental tendon cell and matrix biology, particularly in comparison with other musculoskeletal tissues. Due to its clinical importance and an extent of access to tissue specimens not possible in the medical field, the most completely understood tendons are those of the distal limb of the horse, in particular the injury-prone superficial digital flexor tendon (SDFT). Citations of equine literature are frequent in descriptions of human tendon biology and pathology (Magnusson *et al.*, 2002; Riley, 2005; Lui *et al.*, 2011). The specialized nature of equine digital tendons and certain human tendons essential for efficiency of high-speed locomotion, and the large size of both species, bring the clinical relevance of laboratory rodent and rabbit models of exercise-induced tendon damage into question. In addition to concerns with the principles of replacement, reduction and refinement (the ‘three Rs’), in-vivo investigations of any tendon in any species will not currently allow direct analysis at the microscopical, ultrastructural and molecular levels required for study of microdamage. There has therefore been increasing effort to develop relevant equine cell culture and tissue explant models. This review will cover current knowledge of equine digital tendon biology and pathology with a focus on the tenocyte (tendon fibro-

blast), discussion of the advantages and problems encountered with in-vitro models used to study tendon cell responses to potential pathogenic factors, and indications of the clinical relevance of such work.

## The Importance of Injuries to Energy-Storing Tendons in Large Athletic Animals

Injury to the SDFT, most often involving the metacarpal segment of the forelimb tendon, is one of the most frequent causes of lameness of thoroughbred horses internationally, occurring in 0.6–16.1/1,000 race starts, with a reported prevalence of 11–30% (Goodship *et al.*, 1994; Williams *et al.*, 2001; Kasashima *et al.*, 2004; Pinchbeck *et al.*, 2004; Ely *et al.*, 2009). In a recent survey of National Hunt racehorses in the UK, the incidence of SDFT injury was 0.95/100 horse months (1 month equalling 30 days) during training and 42.5/100 horse months for those in racing (Ely *et al.*, 2009). The risk of SDFT injury has been shown to increase with horse age in a number of surveys (Williams *et al.*, 2001; Ely *et al.*, 2004; Kasashima *et al.*, 2004; Perkins *et al.*, 2005a). In one Japanese study, approximately 70% of 1,200 racehorses that sustained tendon injuries (largely to the SDFT) failed to return to previous levels of performance in any race (Oikawa and Kasashima, 2002). The results of a UK study of performance of 401 horses sustaining an SDFT injury were more equivocal, with no significant post-injury reduction in maximum Racing Post Rating (O’Meara *et al.*, 2010). The absence of an internationally agreed protocol for reporting of injuries and race performance makes it difficult to compare these datasets. Re-injury rates of 23–67% have been reported in various equine sports disciplines; 19–70% are ultimately retired due to the original or the subsequent re-injury (Marr *et al.*, 1993; Dyson, 2004; Perkins *et al.*, 2005b; Lam *et al.*, 2007; O’Meara *et al.*, 2010). All of these factors cause significant economic loss and animal welfare concerns.

### *The Human Element: Tendon Equivalence in Function and Injury Susceptibility*

In the functionally-equivalent human Achilles tendon (AT), increased sports participation in the

Western world in particular is generating a wave of exercise-related injuries. Of total AT injuries approximately 65–75% are sports related; the variety of sports types involved differs between countries. There are also unexplained increases in older non-athletic people (Möller *et al.*, 1996). In Finland between 1987 and 1999 there was an 80% increase in incidence in people in their 3rd to 4th decade (Nyyssönen *et al.*, 2008). In Scotland a 90% increase occurred between 1995 and 2008, with AT injuries comprising >10% of total soft tissue injuries (Maffulli *et al.*, 1999; Clayton and Court-Brown, 2008). In 1986 there was a sudden increase in the incidence of AT rupture in men in Scotland during a year when the Commonwealth Games were held in Edinburgh (Maffulli *et al.*, 1999). The risk of AT injury is associated with elite athletic activity and with age, as for SDFT injury in horses; incidences of up to 29%, 18% and 52% have been reported in elite male runners, elite gymnasts and running athletes, respectively, with the latter figure correlated with hours spent training (Paavola *et al.*, 2002; Knobloch *et al.*, 2008; Emerson *et al.*, 2010). In one study of 785 male former elite athletes, their cumulative incidence of AT degeneration or rupture before the age of 45 was five to eight times that of the control population; however, for AT rupture it was up to 15 times that of controls in sprinters specifically (Kujala *et al.*, 2005). Up to 29% of patients with AT injuries may require surgery (Zafar *et al.*, 2009); time for a return to activity varies from 6 weeks to almost 10 months depending on the complexity and chronicity of the injury, and re-rupture rates of 2–12% have been reported (Molloy and Wood, 2009; Saxena *et al.*, 2011).

The functional equivalence of the SDFT and AT is largely due to their pivotal role in saving energy during high-speed locomotion, by reducing muscular work (Biewener, 1998; Malvankar and Khan, 2011). Most tendons ('positional' tendons) simply connect skeletal muscle and bone, stabilizing and moving joints. In the horse, recovery of mechanical work during tendon elastic recoil (i.e. with the SDFT acting as a 'biological spring') has been estimated at 36% at a gallop; in the human AT, energy returns contribute to over 50% of positive work done at the ankle (Biewener, 1998; Farris *et al.*, 2011). Energy-storing tendons must undergo high strains (i.e. changes in tendon length as a percentage of initial length) to store sufficient amounts of energy. The SDFT and AT have conflicting requirements of elasticity to maximize energy storage and strength to support weight bearing. Comparison of SDFT strains *in vitro* with those in galloping horses indicated very low mechanical safety margins, considered to be typical for such structures (Stephens *et al.*, 1989;

Wilson and Goodship, 1991). It is generally agreed that the levels and most likely also the patterns, types and durations of physical stress on tendons are the most important aetiological factors in athletic injuries (Riley, 2005). In addition to the mechanical environment, the only other directly measured or calculated stress factor specific to energy-storing tendons is hyperthermia. Due to loss of some strain energy as heat (hysteresis), which is not effectively dispersed by the vascular system, temperatures of at least 43–45°C have been measured in the SDFT core of the galloping horse (Wilson and Goodship, 1994). Conservative estimates of hyperthermia in the human AT, calculated by mathematical modelling, predicted a core temperature of at least 41°C during 30 min of treadmill running, with the potential to rise to up to 44°C in some individuals (Farris *et al.*, 2011). Such temperatures would be expected to be lethal or at the very least highly stressful to fibroblastic cells. Other damaging conditions that may be experienced during and/or following exercise within the tendon tissue include hypoxia due to tendon hypoperfusion and increased levels of reactive oxygen species (ROS) induced by ischaemia-reperfusion and/or hyperthermia (Paavola *et al.*, 2002; Bestwick and Maffulli, 2004; Millar *et al.*, 2012).

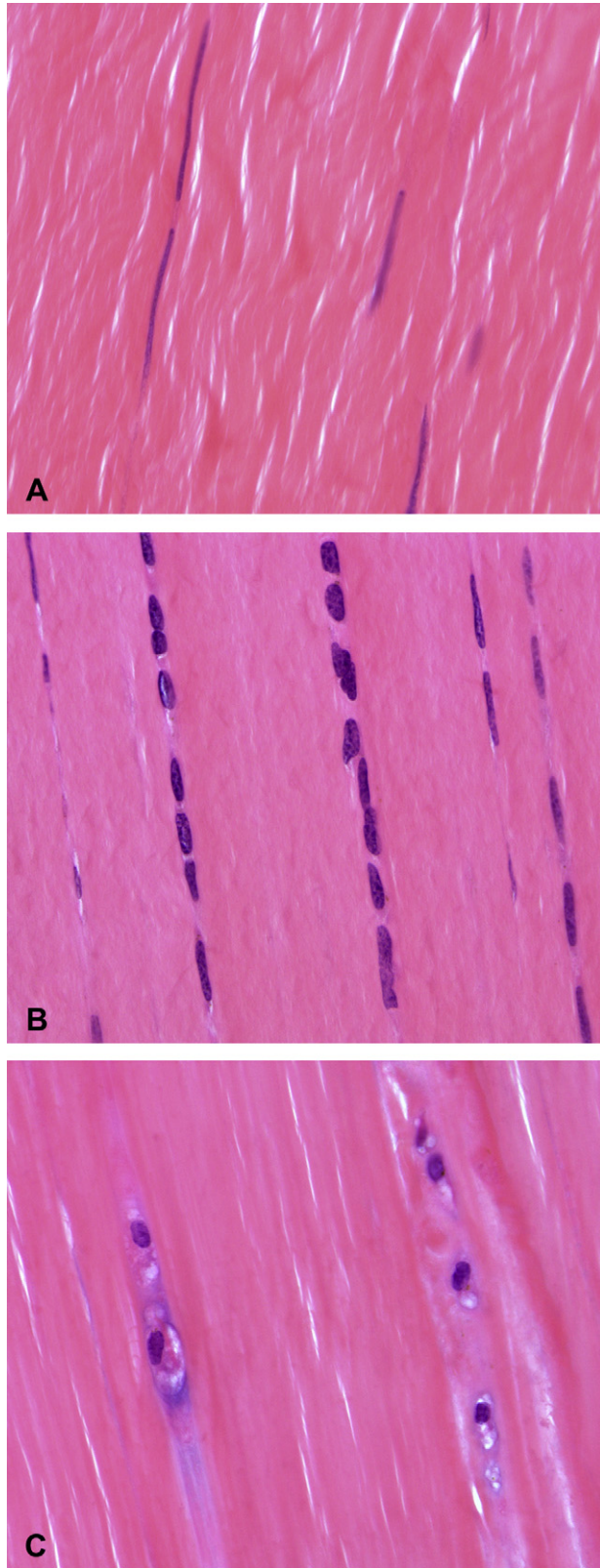
### Tendon Cell Biology

In comparison with other musculoskeletal tissues, the structure and cell biology of tendon tissue is still poorly understood, but has been the subject of increasing research effort over the past few decades. Tendon structure is highly specialized due to the unidirectional forces to which these structures are subjected, and has a complex hierarchical arrangement.

#### *Tenocyte Networks within the Hierarchical Structure of Tendons*

Tenocytes are the fibroblastic cells responsible for synthesis, maintenance and degradation of the extracellular matrix (ECM) of tendons. Their synthetic products include collagen, proteoglycans and glycoproteins, and degradative enzymes including matrix metalloproteinases (MMPs) and their regulators (i.e. tissue inhibitors of MMPs; TIMPs). Despite their pivotal role, numbers of tenocytes in tendon tissue are low, with fewer than 300/mm<sup>2</sup> in the adult SDFT (Stanley *et al.*, 2007). Tenocytes are classified as type 1, 2 or 3 with spindle-shaped nuclei, cigar-shaped nuclei or a chondroid appearance, respectively (Smith and Webbon, 1996; Fig. 1). Type 1 and 2 tenocytes have been equated to 'tenocytes' and 'tenoblasts' in non-equine species; tenoblasts





appear to be more synthetically active and their proportion reduces with age in the SDFT and other tendons (Chuen *et al.*, 2004; JPK personal observation). Type 3 cells are found in fibrocartilaginous areas of tendons where they ‘wrap around’ joints and experience compressive as well as tensile forces. Tenocytes are arranged in widely-spaced rows within the matrix that are bridged by their narrow cytoplasmic processes; these processes and the cell bodies (within rows) are connected by both gap junctions (GJs) and adherens junctions (AJs). Tenocytes are connected to the ECM by adhesive junctions of which the major transmembrane components are integrins (i.e. heterodimeric receptors that physically link the ECM to the actin cytoskeleton).

The (possibly sheet-like) cytoplasmic processes of tenocytes extend around collagen fibrils, the cylindrical units of tensile strength in tendon that are arranged in parallel in the longitudinal axis (McNeilly *et al.*, 1996; Ralphs *et al.*, 2002); collagen comprises 75–80% of tendon dry weight and in the adult 95% of it is type I. Type III collagen is associated with smaller collagen fibril diameters (i.e. weaker fibrils; Birk and Mayne, 1997) and is found in greater amounts in immature and damaged tendons (Williams *et al.*, 1984). Low levels of non-fibrillar collagen types have also been identified. The collagen fibrils follow a planar, zig-zag waveform termed the ‘crimp’, which is thought to act as a mechanical safety buffer (i.e. this waveform opens out under tension before the fibrils themselves are stretched; Wilmink *et al.*, 1991). A mixture of large- and small-diameter fibrils in the SDFT is thought to provide the tendon with strength and elasticity, respectively (Patterson-Kane *et al.*, 1997b; Fig. 2). The remainder of the ECM is comprised of proteoglycans, glycoproteins, small numbers of elastic fibres, ions and water. Tenocytes and their ECM form subunits called fascicles, which measure approximately 1 mm in diameter, are polygonal in cross-section and are surrounded by small amounts of loose connective tissue termed the endotenon (Fig. 3). The fascicles appear to be able to move independently as the tendon is cyclically loaded, with lateral force transmission between them being small or negligible (Goodship *et al.*, 1994; Haraldsson *et al.*, 2008). The endotenon carries blood vessels, which do not penetrate the fascicular substance, in

Fig. 1. Examples of the three morphological tenocyte types reported in equine digital tendons: (A) type 1 cells with long, spindle-shaped nuclei; (B) type 2 cells with plump, cigar-shaped nuclei; and (C) chondrocyte-like type 3 cells, typically found in ‘wrap around’ regions of tendons where the matrix is fibrocartilaginous. Haematoxylin and eosin.  $\times 400$ .

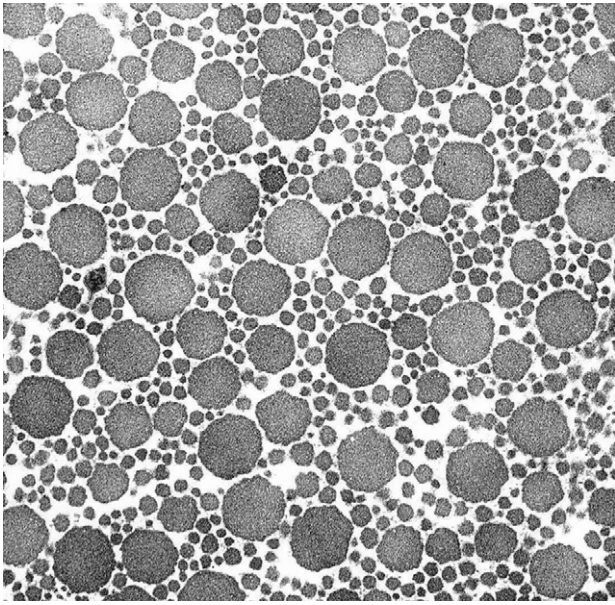


Fig. 2. Transmission electron micrograph of a SDFT from a thoroughbred horse, showing the collagen fibrils in cross-section. The large and small diameter fibrils are thought to provide the tendon tissue with strength and elasticity, respectively. Uranyl acetate and modified lead citrate.  $\times 45,100$ .

addition to lymphatic vessels, nerves and fibroblastic cells; transforming growth factor (TGF)- $\beta$  is only found in the endotenon in the adult SDFT (Cauvin *et al.*, 1998). Fibroblastic cells are also located in the epitenon and paratenon (i.e. connective tissue layers surrounding the whole tendon), but the nature of these cell populations has not been explored in detail.

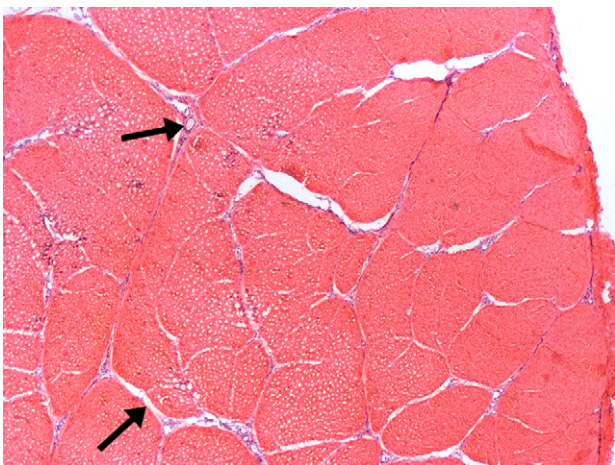


Fig. 3. Cross-section of an equine SDFT, showing polygonal fascicles that are surrounded by a thin sheath of looser connective tissue termed the endotenon (arrows). Blood vessels are only located within the endotenon in non-injured tendon tissue. Cryosection, haematoxylin and eosin.  $\times 20$ .

#### *Modelling Tenocyte Networks In Vitro*

There is a need to use in-vitro experimental systems for studies of tenocyte responses to conditions occurring during exercise. The advantages of such systems are that they are often easy to use, tractable, amenable to biochemical analyses and time-dependent measurements can be made at both cellular and molecular levels. Technical, animal welfare and cost issues prevent the latter in experimental animal trials, particularly if horses are to be used. It is, however, critical that the cellular read-out recapitulates phenotypic characteristics known for the donor tissue; results from many models only have relevance to that particular in-vitro environment. Access to tissue specimens from horses submitted to abattoirs or post-mortem diagnostic services, with owner consent, can significantly facilitate tendon research relative to the medical field. There can, however, be problems in obtaining full histories for the animals, in particular exercise history. In the ideal situation, the extent of pre-existing damage to cells and tissue from each donor should be quantified prior to any manipulation or applied stress.

*Tendon Cell Culture Systems.* Two-dimensional (i.e. monolayer) culture is the easiest starting point for studying tendon cells, however (1) tenocytes in tissue are not arranged in confluent or near-confluent sheets (as above); (2) the severity of methods used to extract them from the tissue can potentially cause serious problems; (3) the process may select certain cellular subpopulations; (4) the problem of phenotypic drift is well-documented; and (5) the relationship between tenocytes and their matrix is critical and difficult to replicate in culture. As tenocytes usually sit in a very small niche with intimate association with the ECM, interference with these interactions by either enzymatic digestion or allowing outgrowth from a tissue fragment (explant) may create injury responses that drive phenotypic drift. We have found that culture following enzymatic digestion of SDFT and deep digital flexor tendon (DDFT) specimens can generate reasonable numbers of viable cells within a week, with slightly more rapid growth from the former tendon. Considerations for monolayer culture in this respect include serum levels, glucose concentration, antibiotic use and oxygen tension. While serum levels of 10% and higher are routinely used, it is clear that this is a non-physiological and potentially damaging mitogenic stimulus. Cell damage may not be evident when using 'binary' live/dead assays, even when considerable levels of genotoxic stress are being experienced and repaired. It is also important to avoid high-glucose media unless there is



a wish to model diabetic pathology, as the effects of this on tendon cells are not well defined. Antibiotics of the fluoroquinolone class have been linked with tendonopathy in man, and can triple the risk of AT rupture (Sode *et al.*, 2007); the pharmacokinetic properties of these drugs promote their concentration in connective tissue. These effects extend to cell culture, with fluoroquinolone treatment of a spontaneously immortalized rabbit tenocyte cell line resulting in cytotoxicity and MMP activation by generated ROS (Bernard-Beaubois *et al.*, 1998; Pouzaud *et al.*, 2004). Enrofloxacin treatment of equine SDFT-derived cells caused altered synthesis of small proteoglycans, changes in cell morphology and inhibition of cellular proliferation; these effects were most pronounced when foal tendon cells were used (Yoon *et al.*, 2004). With the use of ciprofloxacin in the medium, we have not noted any effects on equine tendon cell proliferation or DNA damage. Effects may be both drug and concentration related and should be tested in the specific cell culture system used if their addition is considered to be necessary, for example to remove *Mycoplasma* spp. infection. Ambient oxygen levels are not physiological for any cell type and are particularly likely to be harmful in terms of ROS generation for cells from poorly-vascular tissues such as tendon. These levels of oxygen are considered to be largely responsible for the 'culture shock' experienced by many rodent cell lines, which can lead to their premature senescence (Sherr and DePinho, 2000). Researchers have reported accelerated proliferation and improved viability of neonatal tendon cells when using oxygen tensions of 2–5% (Zhang *et al.*, 2010b). However, robust datasets for cells derived from various tendons of adult animals under these conditions are lacking.

For viability measurements, the colorimetric MTS (3-[4, 5-dimethylthiazol-2-yl]-5-[3-carboxymethoxyphenyl]-2-[4-sulfophenyl]-2H-tetrazolium) assay for metabolic activity may be preferable to other methods as reagent compatibility is not an issue. In general, working with equine tendon cells and tissues can be problematic due to the need to determine reagent and antibody compatibility with this species. We have found, for example that two commercial antibodies to cleaved caspase-3 documented as labelling equine tissue sections by other authors, did not convincingly label equine cells or histological sections. These antibodies were negative in duplicate western blots using staurosporine-treated equine fibroblasts; this occurred despite a high sequence identity with species-matched pre-proteins. Care should be taken in using antibodies and reagents published as reactive for equine tissues or cells if formal validation is lacking. In terms of measuring apoptosis, researchers detecting DNA breaks using

the terminal transferase-mediated dUTP nick end labelling (TUNEL) technique should also be aware of the caveats of that methodology. False-positive and false-negative results have been obtained, but combining this technique with morphological cell count analysis may improve data quality (Garrity *et al.*, 2003).

Monolayer studies must also be analyzed with the understanding that the cells are selected on the basis of their ability to adhere to culture dishes following extraction. The growth of cells from explants, which involves a more lengthy initial recovery period, is also selective in favouring cells that both migrate and replicate away from the injured tissue margins. Growth factors including insulin-like growth factor-1 are often added to media to encourage growth, but they are used at non-physiological levels and may encourage outgrowth of poorly understood subpopulations (Costa *et al.*, 2006). The success of growth factors may in part be due to loss of inhibitory stimuli usually provided by the ECM. Type I collagen, for example, can exert cell cycle inhibitory effects via integrin-linked regulation of mitogen-activated protein kinase activation (Schöcklmann *et al.*, 2000). Another concern with primary cultures from tendon tissue is that there are no specific markers to differentiate tenocytes from other fibroblastic cells originating from the endotenon, epitenon or paratenon. Some researchers have suggested that sequential enzymatic treatment can be used to separate fibroblasts from the epitenon and outer sheaths (Banes *et al.*, 1988); however, there is no way to be certain without appropriate markers in cell culture or *in situ*.

A further well-known problem with tendon cell culture is that within a few passages, substantial phenotypic drift occurs; this is the case for cells from both normal and injured tendons (Schwarz *et al.*, 1976; Bernard-Beaubois *et al.*, 1997; Yao *et al.*, 2006). Documented changes have included increased ratios of type III to type I collagen and reduced expression of decorin; it has been proposed that only cells within the first three passages be used (Mazzocca *et al.*, 2011). This is a difficult situation when trying to obtain sufficient cells from very small biopsy specimens obtained from human patients, but is less of a problem when working with equine models as large amounts of tissue can be acquired, and further specimens can always be obtained.

Many of the above issues in tendon cell culture may relate to failure to provide appropriate extracellular matrices. In two-dimensional culture, this involves providing surfaces that encourage normal cellular behaviour. Collagen type I surfaces are known to be more appropriate than either glass or plastic; however, the random orientation of small collagen fibrils

in this situation provides only a subset of the organizational cues provided in tissue. Methods for encouraging collagen fibril self-organization to mimic that seen in tissue include variations of systems that drain, shear and confine collagen to thin films or microfluidic channels (Lee *et al.*, 2006; Xu *et al.*, 2011). Electrospun collagen, which involves dispensing the protein from a charged tip, has also been used to generate scaffolds for tissue engineering (Matthews *et al.*, 2002). However, the tensile properties of such fibrils and the extent to which they act as true biomimetics of tissue ECM are controversial (Zeugolis *et al.*, 2008; Jha *et al.*, 2011). While an exhaustive description of such methods is beyond the scope of this review, it should be noted that we cannot yet construct collagen fibrils of specified length or thickness. One practical solution in equine research has been to use tendon tissue specimens as surfaces. In one study, mesenchymal stem cells (bone marrow-derived) and SDFT tenocytes seeded onto acellular native tendon matrices (ANTs) aligned in the longitudinal axis (sometimes in rows), down-regulated type I and III collagen gene expression and became more similar to each other than in standard two-dimensional culture (Richardson *et al.*, 2007). The ANTs were created by cutting 70  $\mu\text{m}$  SDFT cryosections and sterilizing them in ethanol prior to rehydration. Culturing tenocytes on surfaces of acellular tendon sections in our laboratory (without ethanol treatment) resulted in similar alignment and a more spindle-shaped appearance.

Many of the above drawbacks associated with two-dimensional culture can be partially mitigated by performing three-dimensional culture in which the tendon cells are seeded into various gels or scaffolds. High density culture in three dimensions has been found to support tenocyte differentiation and repair, reversing the de-differentiation seen in monolayers (Stoll *et al.*, 2010). The emphasis has been on designing cell–matrix combinations for transplantation, with a biodegradable matrix being required to drive cell survival, migration and repair (Cao *et al.*, 2002; Kazimoğlu *et al.*, 2003). Commonly used gel polymers include natural and synthetic hydrogels (Slaughter *et al.*, 2009). Hydrogels are customizable networks of highly water-absorbent homopolymers, copolymers or macromers cross-linked to form insoluble polymer matrices. Natural hydrogel constituents include agarose, alginate, chitosan, hyaluronan, fibrin, collagen and Matrigel; Matrigel is the trade name for a gelatinous protein mixture secreted by a mouse sarcoma cell line. Synthetic gels including various polystyrene formats and poly[lactic-co-glycolic-acid] (PLGA)-scaffolds have also been employed to study the growth and differentiation properties of ex-vivo tenocytes or

stem-like cells. The synthetic versions are advantageous in terms of ease of manufacture and product consistency; however, the chemistries and cross-linkers used in the production of these synthetic gels can lead to concerns over toxic contaminants in the end-product. One problem when using any gel type is that the normal tendon matrix is not as highly hydrated. A promising methodology has involved using plastic compression to drive water from collagen/cell scaffolds in order to generate more authentic tissue biomimicry; there is less than 10% cell death, and the resultant flat collagen sheet is then rolled along its short axis to form a tendon-like structure (Brown *et al.*, 2005). More sophisticated cell model systems employ mixed cellular populations in order to generate composite scaffolds. These are especially useful in studying graft attachment to bone (Spalazzi *et al.*, 2006). Alternatively, tissue explants may be used as experimental models (i.e. retaining the cells *in situ* in their native matrix). Multiple explants of sufficient size (2–3 mm thick) can be obtained from the large equine digital tendons. A major disadvantage is that explants cannot be stored and there is therefore considerable planning and time-pressure involved in these experiments. There will be a healing response that might complicate analysis although in one study it was determined that fibroblastic proliferation and collagen synthesis predominantly occurred at the end of explants and in the immediately subjacent endotenon (Murphy and Nixon, 1997).

#### *Stem Cells/Progenitor Cells in Tendon Tissue*

A significant question, aside from the nature of tenocytes and other fibroblastic cells, is whether stem cells are also present in tendon tissue and how they are affected by exercise and/or microdamage. It has been hypothesized that tendon-specific stem or progenitor cells (TSPCs) are localized in the endotenon, although this has not been proven (Godwin *et al.*, 2012). Progenitor cells within tissues are intermediate between stem cells and fully-differentiated cells; unlike stem cells they can only replicate a limited number of times and tend to be more limited in terms of the cell types into which they will differentiate. However, the terms ‘progenitor’ and ‘stem’ cell are often used interchangeably, reflecting a current lack of understanding of these complex and most likely heterogeneous populations in various tissues. Stem cell properties of cells of both bone marrow and tendon origin have been demonstrated including clonogenicity, self-renewal and multi-differentiation potential; some origin-specific differences in the latter have been noted, with, for example, more robust osteogenesis from TSPCs in several studies (Bi *et al.*, 2007;

Lovati *et al.*, 2011; Rui *et al.*, 2012). Multipotent cells isolated from tendons of rodents, rabbits and people have shown (species-variable) differences in gene expression profile and cell surface marker expression to those of bone marrow stem cells, indicating that they may be identifiable *in situ* (Bi *et al.*, 2007; Rui *et al.*, 2012). In mouse tendon, TSPCs were defined as being slow cycling during a rapid growth period, with retention of bromodeoxyuridine label for 8–14 weeks (Bi *et al.*, 2007). These cells were found within the collagenous matrix rather than the endotenon, and ultimately comprised approximately 6% of the cellular population; it was suggested that in tendons the stem cell ‘niche’ is comprised largely of ECM rather than being perivascular in nature (i.e. within the fascicle rather than the endotenon; Bi *et al.*, 2007). However, the markers identified as being specific for TSPCs in that study were not used to identify them in the tissue sections. Many authors consider the perivascular niche to be more likely, with cultured perivascular cells from human supraspinatus tendon specimens expressing both tendon cell markers (e.g. scleraxis, collagen types I and III) and stem cell markers (e.g. CD133, Musashi-1, nestin, CD44 and CD29); importantly, the stem cell markers and scleraxis were also expressed by these cells *in situ* (Tempfer *et al.*, 2009). These perivascular TSPCs may be pericytic, as they also express  $\alpha$ -smooth muscle actin.

It is not known how TSPCs in specific tendons, including those that are injury-prone, respond to various exercise-induced mechanobiological factors. Results from work using rodent TSPCs suggest that varying levels of mechanical strain may drive these cells down either undesirable or desirable differentiation pathways; as an example of the latter, in one study TSPCs from treadmill-exercised mice showed increased proliferation and there was increased collagen production in mixed TSPC-tenocyte culture, particularly when sourced from the AT, compared with the anatomically opposing patellar tendon (Zhang and Wang, 2010; Zhang *et al.*, 2010a). Although TSPC subpopulations in the SDFT and AT do not effect regenerative repair following rupture, their role may be more important in response to and repair of microdamage. This could include induction of appropriate responses from tenocytes, as the two cell types are likely to be communicating within the tendon tissue (Zhang *et al.*, 2010a). Additionally, studies of rodent tendons indicate that TSPC numbers and differentiation and self-renewal capacities reduce with age, this being a major epidemiological factor in tendon injury (Zhou *et al.*, 2010). In terms of cell culture, physiological (i.e. lower than ambient) oxygen tensions and/or step changes in these may encourage growth of stem/progenitor cells. Increasing

amounts of data indicate that stem or progenitor cell niches are maintained at lower oxygen levels within the tissue (Mohyeldin *et al.*, 2010). One suggestion is that reduced free radical exposure limits stress-induced differentiation, although stem-like cells also appear to express robust ROS quenching capacities. The response to cell culture conditions, in addition to the tissue location, number and age- and tendon-specific repair capacities of TSPCs in equine digital tendons require further investigation. This may be particularly important given that the ECM that putatively forms the stem cell microenvironment shows significant differences between tendons, with age and in response to exercise in horses and people.

#### *Tenocyte Mechanotransduction*

Tenocytes and, as discussed above, TSPCs, are highly responsive to mechanical loading. However, the precise mechanisms by which they sense these signals and transduce them into cellular responses remain a key area of research. Mechanical signals are most likely detected via fluid flow-induced shear stress and matrix-induced deformation of the cytoplasmic membrane, nucleus and/or cytoskeleton and integrin-containing cell–matrix adhesion complexes (Arnoczky *et al.*, 2002a; Wang, 2006). Tenocytes also express a single primary cilium (i.e. a microtubule-based sensory organelle) that projects an axoneme from the cell surface into the ECM and is aligned in the longitudinal axis of the tendon (Donnelly *et al.*, 2010; Fig. 4). These cilia are deflected in response to tensile loading and possibly also fluid

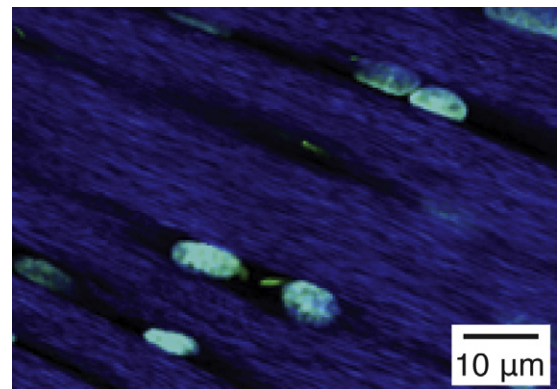


Fig. 4. Image of a tendon fascicle from the digital extensor tendon of a Sprague-Dawley rat showing tenocyte nuclei (cyan), primary cilia (green) and the collagenous ECM (blue). The cilia, extending from surfaces of tenocytes, are aligned in the longitudinal axis of the tendon and are thought to play a mechanosensory role. From: Donnelly *et al.* (2010) Primary cilia are highly oriented with respect to collagen direction and long axis of extensor tendon. *Journal of Orthopaedic Research*, **28**, 77–82. Reprinted with permission.



flow and are thought to play a significant role in mechanotransduction (Lavagnino *et al.*, 2011). In one study of cultured rat tail tendons, the length of tenocyte cilia increased during stress deprivation, but this was reversed by cyclical tensile loading (Gardner *et al.*, 2011). Also, in response to mechanical loading, tenocytes up-regulate n-cadherin (AJ component) and vinculin (linking integrin and AJ components to the cytoskeleton) and assemble more of their actin fibres into stress fibres (i.e. with tropomyosin; Ralphs *et al.*, 2002). Maintenance of cell–cell contact during stretching by these junctional complexes and stress fibres is obviously important for mechanotransduction and possibly also for active recovery during relaxation of the tendon. However, it also prevents mechanical uncoupling of the GJs that are necessary for coordination of tenocyte activity via direct intercellular communication (see below). During cyclical loading, connexin 43 protein (Cx43), a major GJ protein in tendon and many other tissues, has been shown to co-localize with actin; recent work has indicated that the protein drebrin physically links Cx43 to the actin cytoskeleton, in theory acting to stabilize GJs at the plasma membrane in addition to facilitating responses to external signals (Ralphs *et al.*, 2002; Butkevich *et al.*, 2004; Wall *et al.*, 2007a). The AJ and GJ are connected functionally by feedback, in addition to physical links as discussed below (reviewed by Giepmans, 2006).

It has been hypothesized that tenocytes establish internal cytoskeletal tension with a mechanostat set-point that determines whether responses to mechanical loading will be anabolic or catabolic (Lavagnino and Arnoczky, 2005). How the exact tensile strain experienced by tenocytes themselves relates to the strains applied to the ECM as a whole are uncertain, as this appears to be influenced by cellular orientation, cellular stiffness, cytoskeleton organization, subcellular organelles and the type and placement of cell–substrate adhesions, resulting in inter- and intracellular heterogeneity; however, estimates are that the fraction of the applied strain transmitted to cells is at least moderate (Wall *et al.*, 2007b).

#### *Coordinated Signal Transmission: the Gap Junction Connection*

The GJs linking tenocytes allow metabolites, ions including  $\text{Ca}^{+2}$  and small molecules  $<1$  kDa (including inositol [1, 4, 5]-triphosphate;  $\text{IP}_3$ ) to pass directly between the cytoplasm of connected cells, facilitating integration and synchronization of cellular activities (i.e. the transmission of mechanically-activated signalling; Wall and Banes, 2005). In tendons, GJs may also serve the specific function of provision of nu-

trients, as most of the tenocytes are not located closely to the endotenon-confined blood vessels (Tanji *et al.*, 1995). GJs are clustered in their hundreds to thousands in cell membranes to form dynamic GJ plaques. Each individual GJ is comprised of two hemichannels (connexons; HCs) embedded in cytoplasmic membranes of apposing cells that dock to form a tightly sealed channel. The hemichannels are in turn each comprised of six connexin proteins, and Cx43 and Cx32 have been identified as the major isoforms in equine, rat, avian and human tendons (McNeilly *et al.*, 1996; Stanley *et al.*, 2007). Some researchers have also demonstrated Cx26 expression, although in rat tail tendon this was found to be largely cytoplasmic (Maeda *et al.*, 2012). Significant amounts of Cx26 protein have not been identified in equine tendon (JPK and DB, unpublished observation). The cytoplasmic site of Cx43 protein is known to be linked into a macromolecular complex with AJ components that include n-cadherin and catenins (reviewed by Giepmans, 2006). The Cx43 protein is also physically linked to the actin cytoskeleton (as mentioned above) and to the  $\alpha$ - and  $\beta$ -tubulin proteins that form microtubules.

There can be rapid alterations in GJ expression and function, with half-lives of 1–5 h reported in cultured cells and intact tissue and other factors, including pH and intra-cellular calcium levels, determining whether a GJ is open or closed (Segretain and Falk, 2004). The precise manner in which mechanical stimulation regulates the intercellular communication of tenocytes is still poorly understood. Gap junctional intercellular communication (GJIC) is known to be necessary for a number of responses to mechanical loading including contribution to inositol triphosphate ( $\text{IP}_3$ )-mediated  $\text{Ca}^{+2}$  waves and up-regulation of DNA and collagen synthesis; the latter were prevented experimentally in avian tendon cell and tissue culture by chemical GJ blockade (Banes *et al.*, 1999a, b; Wall and Banes, 2005). Conflicting in-vitro results on effects of mechanical load on expression and function of the GJs themselves have been published, which may reflect differences in load regimens, cells or tendons used and species of origin. Avian tenocytes subjected to 0.5% cyclical strain at 1 Hz for up to 7 days up-regulated Cx43 gene expression, and when norepinephrine was also used they increased GJIC (Wall and Banes, 2005). Reduction in Cx43 protein expression was reported in avian tenocytes subjected to discontinuous 0.5% cyclical strain (Banes *et al.*, 1999b). In rat tail tendon fascicles subjected to 1 N static tensile load for 1 h, there was a significant reduction in Cx43 protein expression and GJ permeability that did not occur following a 10 min loading period (Maeda *et al.*, 2012). Similar studies using equine or

human tendon cells or tissue specimens and more physiological cyclical mechanical loading regimens have not been conducted.

*Connexin Isoforms.* Potential differential effects of Cx43 and Cx32 isoforms on tenocyte function also require further investigation. These isoforms do not occur within the same hemichannels (i.e. as heteromeric connexons) and have a different physical distribution, with both linking tenocytes within the same row, but only Cx43 comprising GJs between cells in different rows (i.e. they may link different local cellular networks; Elfgang *et al.*, 1995; McNeilly *et al.*, 1996). There is some evidence that they may modulate tenocyte collagen expression differentially with Cx43 being inhibitory and Cx32 stimulatory (Banes *et al.*, 1999a; Waggett *et al.*, 2006). The latter study does not agree with increased collagen gene (*Coll1A1*) and total collagen protein expression by dermal fibroblasts in acutely Cx43-asODN-treated skin wounds; however, the environment in damaged connective tissue *in vivo* is more complex and included elevated TGF- $\beta$ 1 activation (Mori *et al.*, 2006). In osteoblasts, a Cx43-responsive element in the promoter region of the collagen type 1  $\alpha$ -1 chain has been identified (Stains *et al.*, 2003). The effects of different mechanical loading regimens on specific connexin isoforms expressed by tendon cells have not been determined. Finally, it should be noted that in 'wrap around' regions of tendons where type 3 tenocytes predominate and the expression of GJs is low to non-existent, coordination of their behaviour must occur by other means (e.g. via indirect cytokine and growth factor signalling; Ralphs *et al.*, 1998). This wrap around region of the SDFT is not prone to injury.

### **Comparative Biology of Injury-Prone and Non-Injury-Prone Tendons in Horses**

The comparison of injury-prone and non-injury-prone equine digital tendons has significantly increased understanding of their relative structure and function and the pathogenesis of exercise- and age-induced microdamage.

#### *Functional Determination of Matrix Composition: Energy-Storing Versus Positional Tendons*

The common digital extensor tendon (CDET), which anatomically opposes the SDFT, functions only to position the digit correctly immediately prior to weight-bearing and makes no significant contribution to elastic energy storage. In the SDFT, strains of up to 16.6% as measured in a galloping horse approximate the failure level, as mentioned above (Stephens *et al.*,

1989; Wilson and Goodship, 1991). In contrast, the CDET, as a non-load-bearing structure, is not thought to experience levels of strain greater than 2.5%; as this tendon has a failure strain level of approximately 10%, there is therefore a wide mechanical safety margin, and injury is rarely reported (Batson *et al.*, 2003; Birch *et al.*, 2008a). The DDFT situated immediately cranial to the SDFT on the palmar surface of the forelimb is also not considered to be an energy-storing structure. The DDFT experiences significantly lower mechanical loads, strains and stresses during weight bearing at a walk, trot and canter and is thought to function mainly to flex the digit during the late swing phase (Platt *et al.*, 1994; Butcher *et al.*, 2009). The suspensory ligament on the palmar aspect of the third metacarpal bone is energy storing and is also frequently injured (Williams *et al.*, 2001; Butcher *et al.*, 2009), but as an intraosseous muscle rather than a tendon: it will not be covered in this review. The differing functions of the SDFT and CDET, which have similar lengths, are reflected in their structural and material properties: the SDFT is a stiffer and stronger structure relative to the CDET as it has a greater cross-sectional area; however, the SDFT matrix as a material has a composition that is more compliant and elastic (with a lower elastic modulus) to allow the tendon to deform and function as an energy store (Batson *et al.*, 2003). These properties do show significant variation between individual horses (Birch, 2007). Similar differences in strength and elastic modulus have also been noted between the energy-storing human AT and the anatomically opposing, positional and non-injury-prone anterior tibialis tendon (ATT) (Birch, 2007). Corresponding to the tendon-specific differences in mechanical properties, as would be expected, are very different matrices synthesized by the respective tenocyte populations. The SDFT has a higher content of sulphated glycosaminoglycans (GAGs), collagen oligomeric matrix protein (COMP; a glycoprotein) and water versus the CDET, with similar differences between the human AT and ATT (Batson *et al.*, 2003; Birch, 2007). Although the SDFT and CDET have similar collagen contents (as does the AT versus the ATT), the SDFT has a significantly lower collagen fibril mass-average diameter (i.e. the collagen is organized differently such that it contains a greater proportion of small diameter fibrils; Patterson-Kane *et al.*, 1997b; Edwards *et al.*, 2005). Smaller diameter collagen fibrils have a larger surface area-to-volume ratio per unit cross-sectional area and therefore provide greater elasticity via their greater potential to form interfibrillar cross-links; this prevents fibrils 'creeping' past each other permanently.

*Tendon-Specific Differences in Tenocyte Populations of Immature and Adult Horses: Cellular 'Switch-Off'*

In both the SDFT and CDET, the cellularity reduces significantly during maturation (up to approximately 2 years of age) and then more gradually during ageing; in all age groups the SDFT is significantly more cellular than the CDET (Stanley *et al.*, 2007; Young *et al.*, 2009). Similarly in man, the adult AT is more cellular than the ATT (Birch, 2007). Between 50 days and 1 year of age, the SDFT of a foal more than doubles in cross-sectional area, in association with rapid increases in bodyweight, and requiring a very active tenocyte population (Kasashima *et al.*, 2002). However, a number of studies have shown that in adulthood, the SDFT tenocytes become significantly less active; in one such study the expression of Cx43 and Cx32 proteins, synthesis of total, type I and type III collagens and GJ permeability were shown to reduce significantly with maturation (Young *et al.*, 2009). This was not the case in the CDET, in which expression of Cx43 and synthesis of type I and type III collagen proteins actually increased with maturation and exceeded levels in the (more cellular) SDFT; additionally, GJ permeability was maintained (Young *et al.*, 2009). Other studies have shown corresponding evidence of lower collagen turnover in the adult SDFT compared with the CDET, with accumulation of 'old' collagen, lower levels of MMP activity and lower levels of the cross-linked carboxyterminal telopeptide of type I collagen (ICTP; i.e. a collagen breakdown product; Batson *et al.*, 2003; Birch *et al.*, 2008b). In one study, analysis of aspartic acid racemization of collagen in a group of horses with a wide age range (4–30 years) indicated a mean collagen half-life of 198 years in the SDFT versus 34 years for the CDET (Thorpe *et al.*, 2010). There was significant variation between individual horses in these values. In cell culture, SDFT tenocytes showed age-related reductions in strain-induced collagen synthesis, but CDET cells did not (Goodman *et al.*, 2004). It has been suggested that the SDFT of the adult horse 'switches off' tenocyte activity to maintain the stiffness and elasticity of the matrix within the very narrow optimal limits for appropriate strength and energy storage (Smith *et al.*, 2002). If this is true, it has very serious implications for the ability of the SDFT tenocyte network to respond to the expected higher levels and frequencies of damage than those sustained by the CDET.

### **The Pathogenesis of Exercise-Induced Tendon Microdamage**

If tenocytes and, possibly, other cellular populations did not constantly repair damage, all tendons would

rapidly weaken and rupture (Ker, 2002). However, when microdamage does accumulate there is debate as to (1) the extent to which it is actively initiated and mediated by aberrant cellular activity, as opposed to unrepaired matrix fatigue caused by mechanical overuse and cellular loss, and (2) what the nature of the initial or very early pathology is. Due to the prevailing opinion that the pathology is degenerative, the pre-rupture condition is currently referred to as tendonopathy (tendinopathy) or tendinosis rather than tendonitis.

#### *Evidence of Matrical and Cellular Microdamage*

Studies of racehorses and experimental horses subjected to specific treadmill exercise regimens have facilitated demonstration of subclinical exercise- and age-induced microdamage to the matrix. Many of these changes have been located specifically in the core of the cross-section of the metacarpal segments of the SDFT, where lesions occur most frequently. These have included: age-related increases in type III collagen and partially degraded collagen; increased total sulphated GAGs, type III collagen levels and collagen turnover in red core lesions; exercise-induced reductions in GAG content and collagen fibril mass-average diameter; denaturation and depolymerization of collagen fibres and increased cathepsin B activity; and exercise- and age-related alterations in the collagen crimp waveform (Miles *et al.*, 1994; Patterson-Kane *et al.*, 1997a, b; Birch *et al.*, 2008a; Thorpe *et al.*, 2010). Cell numbers, as measured by quantification of DNA levels, were not shown conclusively to reduce in these areas of microdamage (Birch *et al.*, 2008a). Other measures of cellular death, damage or inappropriate activity have not been made.

Studies using human tissue, in which microdamage is correlated with a well-defined exercise history, are difficult to perform; however, there has been access to ruptured tissue during surgical procedures. This has allowed observation of chronic degeneration (i.e. prior, subclinical pathological change) in recently ruptured tendons; in one large study degeneration was found in 97% of tendon ruptures and, interestingly in 35% of 445 cadaveric 'control' specimens (Kannus and Józsa, 1991). The degenerative changes were more severe in ruptured tendons as opposed to tendonopathy without rupture, supporting the microdamage theory (Tallon *et al.*, 2001). As the equine SDFT is not usually treated surgically, access to ruptured tendons in terms of tissue harvesting has been very limited and similar pathological studies of potentially more advanced degenerative change have not been performed. Degenerative changes



described in human tendons are similar (but not identical) to those found in equine exercise studies and include evidence of new collagen synthesis with increased type III collagen and reduced type I collagen, increases in levels of fibronectin, tenascin C, GAGs and proteoglycans and histological observations of apparently irreversible matrix change including irregular arrangement, altered crimping, disruption and reductions in density of collagen (Riley *et al.*, 1994a, b; Järvinen *et al.*, 1997; Cook *et al.*, 2004). Fibronectin and tenascin C are glycoproteins that are thought to modulate cellular activity and migration (Riley, 2005). Increased amounts of type III collagen at AT rupture sites in one study were not associated with evidence of recent synthesis (cleaved propeptides), supporting the theory that this accumulates prior to clinical injury (Eriksen *et al.*, 2002). Histological degenerative lesions seen in the human AT, but not noted in horses, include mucoid degeneration, fatty degeneration (accumulation of adipocytes), calcification and neovascularization; this may to some extent relate to the fact that they are not identical anatomical structures. In both asymptomatic and painful degenerate human tendons, various histological changes have been noted in tenocyte populations including increased or decreased cellularity, plump ovoid or rounded nuclei with ultrastructural evidence of increased production of collagen and proteoglycans, and chondroid change in more extreme cases; tendon cells cultured from lesions have maintained a higher proliferation rate and produced greater quantities of type III collagen (Leadbetter, 1992; Maffulli *et al.*, 2000; Rolf *et al.*, 2001; Cook *et al.*, 2004). Ultrastructural changes in the tenocytes have been interpreted as indicative of hypoxic degeneration, including alterations in sizes and shapes of mitochondria and nuclei, increased numbers and sizes of lysosomal vacuoles, 'hypoxic' and lipid vacuolation and intracytoplasmic or mitochondrial calcification (Kannus and Józsa, 1991; Leadbetter, 1992). Not all authors agree with the definition of these lesions as degenerate and irreversible, referring to them as a failed healing response; the reality may be that both are occurring or that there is a continuum of pathology from reactive tendonopathy through dysrepair (tenocyte nuclear rounding and matrix production) to degeneration (cell loss and death), at which point reversibility is unlikely and rupture may occur during loading (Cook and Purdam, 2009; Fig. 5).

#### The 'Tendonosis Cycle'

The tendonosis cycle is thought to begin when mechanical overloading and other factors overwhelm

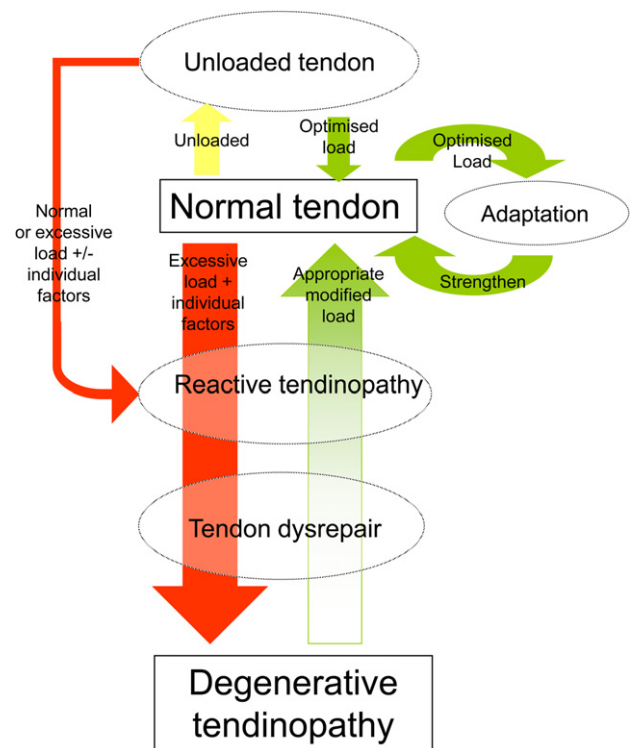


Fig. 5. A proposed model for a continuum of tendon pathology, in which there is a progression from reaction to dysrepair and then irreversible degeneration if endogenous cellular populations do not react appropriately. Not all aspects of this pathway have been identified morphologically in equine tendons. Cook and Purdam (2009) Is tendon pathology a continuum? A pathology model to explain the clinical presentation of load-induced tendonopathy. *British Journal of Sports Medicine*, **43**, 409–416. Reprinted with permission.

cellular repair mechanisms, resulting in increased cell death and potentially inappropriate synthetic and degradative responses that further weaken the matrix (Leadbetter, 1992; Yuan *et al.*, 2002); this does not exclude the contribution of direct damage to the matrix, either in initiation or, particularly, in progression of damage when compromised tissue continues to be loaded. Reasons for inadequate cellular repair may include insufficient time between episodes of exercise, a high frequency, duration and/or magnitude of overuse that simply overwhelms capacity, and individual susceptibility due to differences in matrix composition and/or cellular activity. Alterations in gene, protein and/or enzyme expression levels of various MMPs, TIMPs and ADAM (A Disintegrin And Metalloproteinase) family peptidases have been measured in human degenerate tendon tissue in multiple studies (i.e. there is significant evidence of remodeling of the matrix prior to the onset of clinical symptoms; Jones *et al.*, 2006; Riley *et al.*, 2002). Elevations of MMP-13, MMP-1 and ADAM with Thrombospondin Motifs (ADAMTS)-5 have been

documented in clinically injured equine SDFTs, interestingly with similar but less marked changes in the contralateral, asymptomatic tendon (Clegg *et al.*, 2007).

#### *Studying the Effects of Cell Stress and Damage in Cell Culture*

Mechanistic studies are required to explain the above observations in damaged equine and human tendon tissue. In cell culture systems, determination of the effects of various stressors requires an initially healthy (i.e. non-stressed) population. Importantly, cellular injury in culture systems may not necessarily alter numbers of viable cells, this being the parameter typically measured. Many researchers also use growth rate as an indicator of the health of the system; however, stress can be a potent inducer of replication. It is not entirely clear how tenocytes, which are largely non-replicative in their natural setting, truly respond to the continuous mitogenic stimuli ordinarily provided *in vitro*. Researchers should be aware of this and as best as they can, control for such responses in their experiments by optimizing cell culture conditions. Sublethal levels of cellular stress might for example be measured prior to manipulation, by quantifying ROS generation using 2',7'-dichlorodihydrofluorescein diacetate (H<sub>2</sub>DCFDA).

As tenocytes under normal conditions are subjected to cyclical strain *in vivo* and many researchers have suggested that mechanical overstimulation of tenocytes initiates microdamage, it is critically important to include this factor in culture systems. There is a significant advantage in this respect in using equine digital tendon cells as there is published information on strain levels, frequencies and patterns experienced at various gaits including galloping. This has been obtained from in-vivo strain gauge measurements, in-vitro measurements and modelling studies (Lochner *et al.*, 1980; Stephens *et al.*, 1989; Lawson *et al.*, 2007). Many previous in-vitro studies have not replicated conditions normally experienced by tenocytes *in vivo*, inclusive of physiological levels and durations of cyclical strain (Arnoczky *et al.*, 2007a). It is important to recognize that tenocyte responses to strain can vary according to the tendon of origin, the age of the animal and the differentiation status (Goodman *et al.*, 2004; Zhang *et al.*, 2010a). In one study, differentiation of rabbit AT TSPCs altered depending on the degree of strain; after 4% uniaxial strain at 0.5 Hz for 12 h, the cells expressed more type I collagen consistent with tenocyte activity. However, when that strain level was doubled they became enriched for markers of adipocytes, chondrocytes or osteocytes; lipid production, mucinous matrix formation and cal-

cification have all been documented in human tendonopathy, as mentioned above.

The available devices with which to alter the mechanical environment in cell culture vary in terms of precision, loading modality (e.g. uniaxial strain, biaxial strain, compression) and methodology used (e.g. pneumatic, magnetic, electromagnetic) (reviewed by Brown, 2000). 'Dial-in' strain parameters can be delivered to cells seeded on elastic membranes or in gel constructs. However, over extended periods of time (i.e. high cycle numbers) the reliability with which these strains are delivered can vary. This can occur due to cycle-induced changes in the immobilizing membrane or gel, or irregularities in the gel polymer in which cells are seeded, if three-dimensional culture is used (Bieler *et al.*, 2009). Where three-dimensional culture or tissue explants are used, subjecting these to strain is typically even more technically demanding, in particular due to difficulties in gripping the specimens appropriately. The available apparatus, both commercial and custom-built, all suffer deficiencies in terms of control and measurement of strain. One group has successfully developed an explant model in which SDFT specimens were subjected to 5% strain at 1 Hz for 24 h. The explants, particularly those from older horses, showed diminished mechanical strength that was associated with the presence of viable cells and their MMP activity (Dudhia *et al.*, 2007).

Interestingly, more recent work indicates that it is understimulation of tendon cells and tissue (in rodent and ovine models) that results in rapid up-regulation of expression of various MMPs and, in some models, expression of ADAMTS (Arnoczky *et al.*, 2008; Smith *et al.*, 2008; Thornton *et al.*, 2010); ADAMTS peptidases cleave various proteoglycans. This could be occurring where tendon cells are cultured in static conditions. Mechanical understimulation in the tendon is proposed to occur initially by damage to isolated collagen fibrils during exercise, either at loads approaching mechanical safety margins of the tendon matrix or by localized abnormal loading (non-uniform stress), resulting in disturbance of mechanotransduction mechanisms (Arnoczky *et al.*, 2007a). This loss of cellular—matrix contact would be accelerated by pericellular matrix breakdown resulting from increased tenocyte production of MMPs. Isolated collagen fibril damage has been produced in the laboratory by mechanical overloading of rat tail tendon fascicles, with MMP up-regulation only involving tenocytes directly associated with those fibrils (Lavagnino *et al.*, 2006). Matrix breakdown in stress-deprived rat tail tendons *in vitro* was prevented by treatment with MMP inhibitors (Arnoczky *et al.*, 2007b).

*Contribution of Tenocyte Death to Microdamage: Primary or Secondary?*

Apoptotic cells have been noted in tendonopathy in man (including that of the AT), and in horses with SDFT lesions both with and without a clinical history of injury (Yuan *et al.*, 2002; Hosaka *et al.*, 2005; Pearce *et al.*, 2009). In patellar tendons of human athletes this has been seen against a background of hypercellularity, interpreted in the tendon pathology continuum model as progression from dysrepair to degeneration (Lian *et al.*, 2007; Cook and Purdam, 2009) (Fig. 5). Sparsely cellular tendons such as the SDFT are potentially highly vulnerable to this loss. Mechanical stress-deprivation *in vitro* has been shown to increase caspase-3 expression and apoptotic death of tendon cells (i.e. apoptosis may occur as a downstream event of collagen fibril damage, as described above, through a process potentially involving alterations in integrin-based cell–matrix connections and/or cell shape; Egerbacher *et al.*, 2008). Some researchers have suggested that high levels of cyclical strain cause cell death directly; however, apoptosis in highly strained tendons in in-vitro experiments could also have resulted from collagen fibril damage (Scott *et al.*, 2005). Tenocytes in degenerate human patellar tendon have been reported to up-regulate adrenergic receptors, stimulation of which is another mechanism by which apoptosis might be induced (Danielson *et al.*, 2007). Cyclical strain for 15–120 min (3–9%) was shown to activate stress-activated protein kinases in canine tenocytes 30–120 min later, in a manner that was proportional to the magnitude of the strain applied; these proteins are important upstream regulators of the apoptosis cascade in some cell types, although they did not induce it in this model (Arnoczky *et al.*, 2002b). It is possible that cell death occurs in response to multiple interacting environmental factors in the tendon during exercise including hyperthermia. Canine patellar tendon cells subjected to temperatures up to 42°C showed cDNA fragmentation and caspase-3 activation, with significant increases in both parameters when 9% cyclical strain was added; however, the cells were subjected to these conditions for non-physiological periods of 6 and 24 h (Tian *et al.*, 2004). Our pilot studies using cultured equine SDFT cell monolayers showed a high level of apoptotic cell death from 2 h following a physiological period of heating (i.e. up to 45–47°C over a 10 min period as in the galloping horse) (Burrows *et al.*, 2009). This cell death was largely prevented by chemical blockade of GJs using 18- $\beta$  glycyrrhetic acid, suggesting that GJIC may

spread death signals following exercise-induced heat stress. This concept is not unique to tendon tissue; propagation of ‘bystander damage’ also occurs in focally injured spinal cord tissue and during reperfusion of the ischaemic myocardium; significant improvements in functional recovery following spinal cord injury were noted in one study when anti-sense oligodeoxynucleotides were used to suppress Cx43 expression (Garcia-Dorado *et al.*, 2004; Cronin *et al.*, 2008). Critically, it may not only be intact GJs that contribute to this phenomenon; uncoupled HC can open and allow exchange of ions and small molecules between the cytoplasm and the extracellular environment. Astrocytic ATP release by HC following spinal cord trauma has recently been shown to significantly aggravate secondary injury (Huang *et al.*, 2012). There has been no detailed investigation of HC function in tendon tissue or cell culture, although we have seen evidence of their presence on occasion in equine SDFT monolayers (JPK/DB personal observation).

*Is the Initial Lesion Inflammatory or Degenerative?*

Few researchers have identified inflammatory cells in tendonopathy of human patients either histologically or ultrastructurally (Józsa *et al.*, 1982; Leadbetter, 1992; Aström and Rausing, 1995; Järvinen *et al.*, 1997). Where they have been noted, for example as infiltrates of neutrophils, or lymphocytes and macrophages, they have been interpreted as part of a healing process following matrix disruption and/or tenocyte death that may precede rupture (Cetti *et al.*, 2003). As a result, tendon microdamage has frequently been proposed to be primarily degenerative. The apparent lack of inflammatory cells, however, may be due to failure to visualize the very earliest lesions, with subsequent observations of progressive degeneration actually being secondary to ineffective healing. In a recent study of subclinical tendonopathic lesions in human subscapularis tendons, macrophages and mast cells were noted in significant numbers, although it is not certain at which stage of development these lesions were (Millar *et al.*, 2010). In people, tendonopathy may be seen in conjunction with inflammation of the surrounding paratendon (Järvinen *et al.*, 1997).

The failure to see inflammatory cell infiltrates also does not preclude inflammatory mediators being involved in a primary fashion, for which there is now considerable evidence. In-vitro, human and rabbit tendon cells and avian digital flexor tendon explants have been induced to produce various combinations of cyclooxygenases, prostaglandin E<sub>2</sub>, phospholipase-A<sub>2</sub>, interleukin (IL)-1 $\beta$ , IL-6 and nitric oxide (NO) in



addition to MMPs, by application of cyclical strain or fluid-induced shear stress (Skutek *et al.*, 2001; Archambault *et al.*, 2002; Wang *et al.*, 2004; Flick *et al.*, 2006). Overexpression of nitric oxide synthase (NOS) has been demonstrated in Achilles tendonopathy (Kane *et al.*, 2008). Hypoxic tenocytes in early human tendonopathic lesions (as demonstrated by increased expression of hypoxia-inducible factor 1 $\alpha$ ) showed up-regulation of IL-6, IL-8 and monocyte chemoattractant protein (MCP)-1, type III collagen and some key mediators of apoptosis (Millar *et al.*, 2012). Equine SDFT cells heated to 40–45°C up-regulated tumour necrosis factor (TNF)- $\alpha$  expression, but only after non-physiological periods (30–60 min; Hosaka *et al.*, 2006). In human subjects exercising for prolonged periods (30 min), accumulation of inflammatory mediators including prostaglandin E<sub>2</sub> and thromboxane have been measured in peritendinous tissue (Langberg *et al.*, 1999). It should be noted that stress deprivation of rat patellar tendons and ATs *in vivo* also resulted in increased expression of cytokines by tenocytes (IL-1 $\beta$ , TNF- $\alpha$  and - $\beta$ ), in keeping with the above theories on inappropriate cellular degradative activity in response to reduced mechanical stimulation (Uchida *et al.*, 2005; Lavagnino *et al.*, 2006; Wang *et al.*, 2010). Pro-inflammatory cytokines such as IL-1 and TNF are able to stimulate MMP expression and activity (Riley, 2005). Cytokines cannot always be presumed to have a negative effect, however, with results of one study of human tenocytes in three-dimensional culture showing that IL-1 $\beta$  up-regulated Cx43 and improved cell survival; GJs may transmit 'survival signals' rather than death signals under certain circumstances (Qi *et al.*, 2011). Similar studies have not been conducted using equine tendon cells or tissue.

### Conclusions

Exercise-induced injury to energy-storing tendons of horses and people occurs at a similarly high frequency, with failure of tissue regeneration contributing to high re-injury rates or retirement from athletic activity. There is now increasing research focus on the role of the tenocyte in accumulation of the matrix and cellular microdamage that predisposes to tendon rupture. Access to equine tissues and the ability to make measurements *in vivo* have already significantly improved our knowledge of the composition, structure and mechanical environment of injury-prone and non-injury-prone digital tendons over a wide age range. The imperative now is to develop tractable cell culture models using this species in order to study stress-induced damage and dysfunction; these models will facilitate a more authentic replication of the *in vivo* microenvironment.

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