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Editorials

Science-in-brief: What is needed to prevent tendon injury in equine athletes? A conversation between researchers and industry stakeholders

Introduction

Superficial digital flexor tendon (SDFT) injury affects up to 30% of Thoroughbred racehorses and other high-level equine athletes and is both exercise and age related; high incidence, prolonged periods out of work, high retirement rates and animal welfare implications are continuing significant problems for equine industries [1,2]. The SDFT is an energy-storing structure essential for efficient high-speed locomotion, with narrow mechanical safety margins. Our understanding of pathophysiology has advanced significantly, but preventative measures have not been developed. Most injuries occur during athletic activity following undefined periods of matrix microdamage accumulation that is not repaired (and possibly directly contributed to) by tendon fibroblasts and other endogenous cellular populations. Detection and/or prevention of the earliest phases of pathology are likely to have a greater effect than improvements in therapy, given that tendon is a slowly healing and poorly regenerative tissue.

This workshop was held in October 2012 at the University of Glasgow, UK to bring a key group of scientists from different disciplines (including cell biology, pathology, bioengineering and physics) together with industry end-users, including veterinarians and trainers. We had a particular focus on the Thoroughbred racehorse and the cell biology of normal and injured tendons. The workshop was funded by the Biotechnology and Biological Sciences Research Council (BBSRC) under the International Workshops scheme.

Discussion focused on three main questions.

- What would trainers find useful and practical in terms of preventing SDFT injury?
- Are there available or emerging technologies that could assist in detection, understanding and prevention of early exercise-induced pathology?
- What are the best cell culture models to test preventative mechanisms in the laboratory, and how can this be extrapolated back to the living tissue?

The following is a summary of the discussion points and review of key concepts, including indications of areas in which significant progress is required.

At the track

It is recognised that racehorse trainers differ significantly in their approaches, attitudes and situations, as is reflected by variation between them in incidences of tendon injury [1,2]. This is a complex issue, but a large component of the variation relates to the type of racing (e.g. National Hunt vs. flat racing), including the ages and 'types' of horses involved and, potentially, the progression and/or nature of the pathology itself. In general, J. Givens (Thoroughbred racehorse trainer and veterinary graduate) felt that trainers would be far more interested in measures to prevent SDFT injury than to treat it, due to the significant effects on athletic careers. This is because there is clear recognition in the industry of the binary nature of this condition, i.e. a tendon cannot be 'a little bit' damaged; however, the situation may be different for owners using horses for recreational purposes/companionship. Any preventative measure that would significantly reduce injury risk (e.g. by 70%) would be likely to be adopted. It also needs to be kept in mind that the SDFT is not the only injury-prone structure to be concerned with, and that there are significant commercial pressures to develop horses and allow them to achieve their athletic limits.

What increases the risk of tendon injury, from the trainer's perspective?

Many factors that might be important in determining injury risk have not been studied in detail, or the information has been conflicting. These factors include track surface conditions, the speed achieved (higher being more risky), the level of 'fitness' of a horse and its conformation. Data on relationships between track surface condition and/or type and racehorse injuries are incomplete and often conflicting. This is, in part, due to the very complex interactions between surface type (e.g. wood chip, waxed), the time taken for it to 'bed in', its age, amount of usage, amounts of organic matter that become incorporated (into artificial surfaces), the level of maintenance (which can be highly variable) and ambient conditions (e.g. temperature, humidity) [3]. It is possible that if the training surface is highly consistent, it will not be seen to play an important role in differences in SDFT injury risk (between horses). However, racing itself occurs on turf in the UK at least (and some horses train on it), and its condition is enormously variable. We do have some information, e.g. a higher risk of tendon injury in the UK has been associated with firmer racing surfaces and with racing in the summer [4,5]. Slipperiness is another factor that may be more important on turf racetracks, where water is applied to achieve consistent 'going' conditions and is not really replicating rainfall (i.e. creating variable surfaces). Where synthetic surfaces are used during training or racing, high or low temperatures can alter these surfaces and the associated racehorse speeds (e.g. by thermal transformation of wax) [6]. Additionally, we know that the SDFT core becomes hyperthermic during galloping due to loss of some energy as heat during elastic recoil [7]. Insulation of the limb might add to this thermal burden. Core hyperthermia of the SDFT in relationship to environmental temperatures and limb insulation has not been measured.

In fact, a large number of very complex horse and management factors interact with external conditions. It is extremely difficult to relate tendon injury risk to previous training and racing history, because better horses tend to race less frequently (4–5 races per year), while the less 'talented' could race up to once a week. Simple comparisons of 'good' and 'average' horses are difficult, because a horse that is racing more often is likely not to be trained as hard. This is additionally complicated by differences between racing and training; speeds at which horses run will often be higher on the racetrack. This means there is a possibility that most SDFT microdamage is accumulated during racing, and also that training exercise may not be sufficiently intense to induce injury resistance (if such adaptation is possible). Other horse factors, including conformation, are difficult to assess objectively, but there are some clues, e.g. SDFT injury risk has been associated with increased metacarpophalangeal angle [8]. Factors that seem to be important for human athletes, such as 'running style', are difficult to measure or change in horses.

What might be done to prevent tendon injuries?

Injuries to the SDFT are frequently noticed 6–10 days following racing, the evening after the horse resumes training. It is possible that many lesions are initiated during a race (or an episode of training in which similarly 'stronger' exercise is undertaken), but develop further to a grossly observable level with some further activity. Detection relies on personnel feeling for swelling; however, while some gains could be made by providing education, this is a very advanced stage of injury, in which some microdamage has already occurred.

Imaging of preclinical changes: A key problem in all research approaches, including those discussed below, is the detection of subclinical pathology

in its very earliest stages, which requires: 1) affordable, noninvasive and objective detection methods; and 2) a means of their application by veterinary surgeons, which may require a change in culture such that fees are based on a 'herd health' approach. Significant amounts of work would still be required to link factors responsible for lesion development with measures to prevent their further progression or to reverse the pathology (if that is possible). These might include training regimens, optimal surface choices and correct frequency of racing. The implementation of some practices, e.g. application of cold boots following exercise (if that were proven to be preventative), might be impossible for every horse in the training/racing environment. In terms of reversibility of injury, trainers and veterinarians often notice transient SDFT swelling in young horses. We need a better understanding of whether this is a different form of pathology (i.e. not chronic and progressive degeneration), why it subsides and/or if the risk of SDFT rupture is higher later on.

Individual optimisation of training regimens: Exercise specifically to induce SDFT adaptation has been a research focus, particularly early in life, although there has been debate about sufficiency levels/intensity of exercise provided to experimental animals, including foals and racehorses. Adaptive change has not been found, other than a more rapid growth in SDFT cross-sectional area, but there was also no evidence of pathology; additionally, there was a later occurrence of musculoskeletal injury (in general) once these horses were in the racing environment [9–11]. Further study of early exercise would require interaction of researchers with stud farms as well as with the trainers. Once horses are in the yard, there are few data available, and it is not certain when horses should ideally start racing or what happens in response to different exercise regimens. It is possible that tendons cannot adapt at all. This is complicated by high individual variability (e.g. in SDFT cross-sectional area, to which relative contributions of genetics and exercise are not known) and our lack of knowledge regarding the most important elements of exercise. For example, the results of recent work using human subjects suggest that low-volume but high-intensity exercise is sufficient for aerobic fitness, although admittedly, the musculoskeletal effects are not known [12]. There may be a delicate balance, because in some studies the total amount of high-speed exercise during training has been associated with increased risk of injury [13]. Maintaining a certain frequency of exercise may also be important. This is supported by findings in human athletes, in whom any return to training requires correctly spaced exercise of appropriate intensity, i.e. 'tendons don't like rest or change' (Jill Cook, cited in [14]). In terms of change, it is important to know whether training on one surface vs. a variety of them is advantageous or not. Other issues include weights carried due to handicapping, jockey weights, tack weights and the more esoteric issue of mental attitude ('the art part of the art and science of training').

Genetic testing and biomarkers: At least some breeders would be likely to use tests that genuinely indicate injury risk. However, much of the variance in the likelihood of SDFT injury is probably not genetic. Biomarkers indicating early pathology would have a far greater appeal for the industry due to the practicality vs. imaging (as frequent scanning of all tendons is not realistic) and the minimally invasive nature of blood sampling. In this respect, one could envision biomarker analysis in the context of a 'stress test' (i.e. pre- and post exercise sample comparison), with equine-specific protein arrays and a system such as the Luminex platform [15]. In this way, a horse can act as its own control, which is a situation that may negate aspects of genetic variation.

How could cooperation with trainers be obtained, for research with impact?

Most of the practical problems involved in developing realistic preventative measures relate to this high-pressure environment, including owner attitudes; the shorter career for flat-racers can be a particular issue if, for example, rest periods are required. Another possibility in terms of practicality could be to employ modelling techniques (for which some data already exist) to identify individuals at increased risk of SDFT injury, and then focus resources and time on those animals.

Studies of training regimens: Trainers are not going to standardise training regimens to test their effects; however, developments in global positioning system (GPS) and various portable monitoring devices (e.g. heart monitors) will now allow more accurate measurement of the training effects. Practically, researchers will need to support regular inputting of these data into databases and analytical software. Other aspects, including weather and track surface conditions, would also need support, i.e. in order to generate any meaningful data on training vs. SDFT injury that takes into account the various complexities introduced above, there will need to be large-scale, researcher-staffed studies that do not interfere with the day-to-day activities in training yards.

Biomarker development: This is an area in which trainers would be likely to participate, particularly if the trials did not involve an increased cost of routine blood sampling. If researchers could provide potentially good markers for early injury, there is likely to be cooperation in sampling those horses in which the work rate is being 'stepped up' and to alter training regimens in horses where early damage is indicated. The potential reversibility of early injury is of great interest to the industry. The onus is on researchers to provide useful candidate markers, and this is an active area of research. The challenge is to validate markers reflective of changes specifically in the SDFT, in a background of products from all of the other tendons and connective tissues. In all likelihood, biomarker analysis in the future will be part of a 'phenotype' put together from imaging + biomarkers + aspects of the physical examination.

But what happens if injuries are discovered?: There is a major issue in conducting this research, in terms of the implications (perhaps in future terms) of whether subclinical injury should be reported. There would be resistance to open reporting of this type of information in many countries. In Hong Kong, mild tendon injuries are now reported to the public, and the result of this was more rapid intervention to deal with early injury. This led to a 20% reduction in retirements from racing due to tendon injury. It might be very positive for equine welfare and public engagement if there was a more open policy; this raises the question as to whether prevention research should be conducted in countries where that attitude exists and/or greater funds are available within the industry.

Horse-side

There are significant gaps in our knowledge of what occurs within injury-prone SDFT sites (i.e. the metacarpal core) during exercise, how this is altered by the multiple external and internal factors discussed above (including exercise type, intensity, duration and frequency), how/why early pathological change develops and whether (up to a certain point) the pathology is reversible. This information is clearly required to develop scientific rationale for preventative methods, both at horse level and in our laboratory models.

What should be measured?

Structural changes in the SDFT prior to macroscopic swelling will most probably represent late phases of subclinical damage, but do require definition in terms of understanding the full spectrum of injury. In terms of the tendon environment, hyperthermia was measured in the SDFT core in one study but not analysed any further in relationship to various environmental, exercise and horse-related conditions. Another important factor never directly measured is the level of oxygen; tendon is not highly vascular (relative to other tissues). Tendons are comprised of fascicular subunits, movement between which is an important determinant of elasticity of this energy-storing structure [16]. These fascicles are formed by collagenous matrix (and lesser amounts of noncollagenous matrix) that is synthesised and maintained by the widely separated and longitudinally oriented rows of tenocytes. The vascular supply is located in loose connective tissue (the endotenon) that separates fascicles, i.e. most tenocytes are not directly adjacent to any blood vessels. Exercise-induced hypoxia or ischaemia-reperfusion has frequently been proposed as a factor in tenocyte damage and death, due to generation of reactive oxygen species, high levels of which lead to oxidative stress [17]. Hyperthermia also increases levels of reactive oxygen species. Reported effects of hypoxia on cultured tendon cells have been highly variable, i.e. apoptotic

cell death [18] vs. high tolerance (even of anoxia). However, we have not been able to plug real-life oxygen and temperature levels into these systems. The responses of tendon cells to tissue conditions that occur during exercise/damage (as well as a better idea of what these conditions are) also require further analyses if we are to validate laboratory models and study mechanisms. These models should include synthesis of collagenous and noncollagenous matrix components. We know, for example, that amounts of *type III* collagen (which forms weaker, small-diameter fibrils than the usually predominant type I) can accumulate in the later stages of preclinical damage in the SDFT core [19]. However, this raises a significant problem for researchers in terms of what cellular populations to monitor and how to do so. There are different populations of fibroblastic cells within fascicles (the tenocytes), between fascicles in the endotenon and within sheaths (the epitenon and paratenon). The paratenon is a loose external sheath in areas where there is no external synovial lining, including the metacarpal region of the SDFT. The paratenon is most easily accessible and is known to contribute cells that proliferate and migrate into injured tendon substance [20], but we do not know whether or to what extent this occurs in early subclinical injury. Mechanical factors are significant drivers of cellular phenotype; cells in the dynamic endotenon environment between sliding fascicles are likely to have very different turnover and activity levels from tenocytes that sit within a narrow niche inside dense, longitudinally oriented collagenous matrix. Levels of ^{14}C in the Achilles tendon of people alive during nuclear bomb tests (1955–1963) indicated that the tendon core is essentially not renewed following maturity [21], and this correlates well with an estimate of collagen half-life of 197.5 years in the equine SDFT [22]. However, the thin endotenon layers have not been analysed separately. We know, for example, that noncollagenous matrix components (e.g. glycosaminoglycans, proteoglycans) alter with exercise in the SDFT [23], and it is possible that this is occurring specifically within the interfascicular compartment [24]. Lubricin is a glycoprotein found to be important in interfasicular movement and surface gliding in other species [25], but it has not yet been investigated in the horse, nor have other cell types, including endothelial cells, immune system cells (e.g. mast cells), elements of the neural system containing neuropeptides (e.g. substance P) and stem cells (tendon or tendon sheath origin).

How do we make these measurements?

The subjectivity of ultrasound image interpretation is a problem, and there are other significant limitations, including resolution that does not extend beyond relatively large structures, such as fascicles. Not every change will be observable, e.g. a tendon core containing significantly elevated amounts of *type III* collagen can have ultrasonographically (and even histologically) normal fibre alignment. However, there are significant advantages to being able to image the whole tendon, and there have been recent technological improvements, i.e. the ultrasound tissue characterisation technique, which has been used to detect early pathology in human tendons and reversible post exercise changes in Thoroughbreds 1–2 days post racing [26,27]. No ultrasound tissue characterisation–histological correlation has been performed using horse tendons. The use of magnetic resonance imaging has been more controversial due to lower structural resolution, but there is at least the potential to pick up subtle change, and improvements in software are occurring that may facilitate this. Power Doppler has already been used to monitor blood flow velocity in the human Achilles tendon during exercise [28]. Photoacoustic imaging has been used to monitor microvascular changes in healing Achilles tendons of mice [29], but has not been applied to larger animal models or preclinical phases. This involves using a pulse laser source to excite the tissue, which then generates ultrasonic waves due to thermoelastic expansion. When considering the whole tendon, biomechanical approaches provide further noninvasive possibilities. However, although various combinations of techniques, including force plate measurements, kinematic analysis and dynamometry, have been used to monitor healing equine and human tendons, their applicability to the detection of early pathology is uncertain. Biomechanical normality does not imply structural and functional normality; in part, this can be due to the adaptation of other structures in the limb.

For measurement of oxygen levels, the blood oximetry technique is one possibility [30,31]. The technique will work through skin, involves transmitting and receiving probes that can be as small as 1 mm^3, and has been used to assess the human Achilles tendon [32].

Monitoring cellular activity, particularly in different tendon compartments, raises further technological challenges. Positron emission tomography involves detection of γ-rays emitted indirectly by a tracer that is injected into the body, such as an analogue of glucose (fludeoxyglucose) to measure tissue uptake; imaging is frequently achieved using a computed tomography or magnetic resonance imaging system. Various markers could be used, including those relevant to hypoxia and inflammation. While expense currently limits its use in horses, there has already been interaction between veterinary researchers and scientists/medical clinicians with access to such facilities. For more direct measurement, it is obvious that the paratenon can be accessed with relative ease. Significant exercise-induced changes in *type I* collagen turnover were measured in the paratenon of the human Achilles tendon using a minimally invasive microdialysis technique [33] that would be easily applicable to horses. Other compartments could only be accessed with significant tissue damage, and their investigation is likely to be limited to *in vitro*/*post mortem* studies.

What about biomarkers – 'the holy grail'?

This was clearly expressed as an interest for trainers, and would potentially avoid the difficulties of imaging. As yet there are no clear individual candidates. Cartilage oligomeric matrix protein [34] and neoepitopes (cleaved matrix proteins generated during injury) are of use only when measured in synovial fluid adjacent to a clinical injury, i.e. they are not applicable to subclinical metacarpal region SDFT pathology. The approach of measuring multiple blood markers simultaneously to obtain a 'phenotype' is more likely to be useful, but there is still the potential problem of tissue specificity (discussed in 'Biomarker development'). Multiplex assays that allow simultaneous measurement of multiple analytes (nucleic acid or protein based) in a single run may increase the affordability. Potential difficulties include optimal storage requirements (-80°C without repeated freeze-thawing), regular sampling of horses and availability of reagents for equine use (researchers have to routinely perform such validations). It should also be kept in mind that predictions of injury risk are likely to lose accuracy when applying these data to individual horses. Greater advances have been made in the medical field in this type of profiling, e.g. using serum and synovial levels of multiple cytokines to identify early stages of osteoarthritis [15]. There is some recent information on the relationship between genotype and tendon injury, but it is not currently of practical use. We do not yet know why (in horses and humans) there is a higher injury risk associated with certain polymorphisms in the minor matrix components collagen *type V* and tenascin [35].

In the laboratory

Given the current limitations in technology and biomarker information, in particular at the tendon compartment/microscopic level, there is a necessity for *in vitro* work to provide scientific rationale for industry involvement in the development of preventative measures. However, there are significant problems with the relevance of systems used in our laboratories, due to the complex multilevel structure of tendon tissue and the environment that it experiences during and after high-speed exercise. Results can also be difficult to relate back to the tissue, with problems in accessing sufficient numbers of *post mortem* samples from horses with defined exercise/racing histories and in consolidation of expertise to conduct appropriate analyses.

What is currently limiting the relevance of our cell culture models?

The very different arrangement of cells in tendon tissue vs. the culture dish is a major problem. In tissue, the tenocytes are arranged in rows within large amounts of matrix, and there is still some confusion as to what their cytoplasm looks like in 3 dimensions (largely not seen histologically), i.e. in the form of sheets or fine processes. This has also not been defined in immature and injured tendons (cell rich) vs. normal adult tendon (cell poor). We are also not certain what cellular populations are derived when

tendon specimens are digested or when cells are grown from explants. There is no specific marker for tenocytes; they do express scleraxis (a marker of tendon development), but the same marker is upregulated during injury by other fibroblastic cells (and digestion and cutting of tissue are both injury events). We do not know how mixed the original cells are, and what we are selecting for in cell culture conditions (particularly with repeated passaging). It is possible to separate the paratenon and epitenon from the tendon substance, and differences in collagen I and III synthesis have been found in other species between cultured tenocytes, epitenon fibroblasts and synovial cells. These cellular compartments also require investigation *in situ* in tissue specimens (e.g. matrix gene and protein expression) to determine whether the differential phenotype is maintained in culture conditions. There is also some confusion as to whether tendon-derived stem cells are being isolated. Multipotent populations have been isolated [36], but even tenocytes *in situ* show phenotypic plasticity in response to damage and/or mechanical environment alterations. The relationship of these events to the cell culture environment and the disease status of the original tissue has not been investigated. For example, synovial mesenchymal stem cells/progenitor cells from human patients with osteoarthritis do not show self-aggregation due to cytokine exposure, including macrophage chemoattractant protein-1 [37]. This comes back in a circular way, to being able to define early pathology in SDFT tissue used for laboratory studies. Findings for human and sheep stem cells [37,38] support the point that perhaps tendon stem cells cannot function well in the injury environment. Control of this tissue environment may be required to realise the full potential of endogenous or injected mesenchymal stem cells/progenitor cells in optimising repair.

The problems with cell culture laboratory dogma

Standard approaches used in cell culture laboratories are not ideal for most cells, but tendon cells may be having more extreme responses to the cell culture conditions, perhaps due to their origin from a particularly highly specialised tissue environment. Although 21% oxygen is typically used, most cells in culture are damaged by it because tissue levels are ~2–7%, with tendons likely to be towards the bottom of that range. Reduced oxygen levels can be achieved using extremely inexpensive systems [39], with 5% being a 'safe' level for tendon cells. There are also problems in terms of assessing stress in cells due to abnormal cell culture conditions and how these interfere with studies of natural injury-inducing factors found in tendons (hypoxia, hyperthermia, mechanical strain and cytokines). A panel of markers for horse cell stress responses is not currently available for interrogation in various systems and conditions, for relating back to normal and injured SDFT tissue. Promyelocytic leukaemia protein is a potential candidate for the central coordinator of stress protein responses. In humans it is complex, with at least 20 isoforms; it has not been studied in the horse. Most researchers do not monitor cell stress in their systems, with assumptions that continued viability is sufficient. It can be monitored very simply by immunolabelling for gammaH2AX, a histone protein that responds to DNA damage. Cell stress is often seen as DNA damage even if this was not the primary injury [40]. The reversibility of stress responses is highly variable, but dependent on the stress type, cell type and cell culture 'ageing' effects (due to expansion, not the age of the source animal). A further important consideration when assessing cell stress responses is that the cells are a population and will not all respond in the same way. Variation may also relate to the point in the cell cycle when they are being observed. It is therefore important to capture all data and not to eliminate 'outliers' as statistically inconvenient [40]. The presence of stem cells will make any variation even more extreme, because they include subsets with differential responsiveness at many levels to multiple factors; while this diversity is a 'complication' when trying to obtain data, it is most likely to be a strength within the tissue, just as it is for immune system cells.

Most work on cell stress using nontendon cells has not focused on effects of the mechanical environment, extracellular matrix composition, matrix stiffness and morphology, or interactions of those factors with cytokines. Tendon cells are significantly damaged when grown on glass or plastic that has not been matrix coated. There has also been no study of the relationship between nuclear shape and stress. Nuclear shape is altered by cyclical deformation, is known to be different in degenerating/microdamaged tendons [41], and influences gene transcription and epigenetic control mechanisms. Some of these problems in the 2D system might be mitigated by growing cells on (cryosectioned) acellularised tendon matrix from the same or a different horse; they attach, proliferate, migrate at least a short distance into the matrix, elongate along the fibres, and have collagen gene expression levels more comparable with cells in tissue [42].

Would 3D systems be better?

The advantage of 2D systems discussed in 'What is currently limiting the relevance of our cell culture models?' is that they are easier to establish, manipulate and (usually) image, but are really only of value for screening, before confirming any findings in more complex (i.e. relevant) models. The problem is that in tissue, the cells are found in multiple compartments in a multilevel structure (including fascicles and fascicular bundles) with other cell types; many aspects of the structure and cells are likely to differ significantly between individuals, with age and in different injury states. This may include the size and nature of the cellular networks. We cannot replicate that with 3D cell-matrix constructs, but we can provide 3D rather than one-sided engagement of the focal adhesion kinase system, more realistic relative amounts of matrix and (potentially) seeding with multiple cell types (e.g. tendon cells with mast cells or macrophages). Bioartificial tendons can be made with relative ease [43]. Another method involves mixing tendon cells into collagen or fibrin gel and allowing them to contract the matrix [44]. The remodelling is reliant on endogenous loading by fibroblasts; it is matrix metalloproteinase dependent, and the matrix has a more 'natural' organisation. Such systems have not been created and compared using cells from the SDFT and other digital tendons for validation; phenotypic differences of SDFT and common digital extensor tendon cells have already been demonstrated *in situ* and in monolayer (2D) culture [45,46].

A further option is to use tissue explants. These have been used successfully to address questions in a way that would not be possible with matrix-free cells [47–49]. However, a significant disadvantage is that they are injured on removal. Some of the explants die very rapidly, while in those remaining viable, there will be cell death on the outside due to trauma. Gene expression alters markedly, and it is not certain whether this returns to normal levels. The thickness of explants is limited to <2 mm due to perfusion limitations. There is an advantage to the low metabolic rate in this tissue, in that when it is kept chilled (4°C) for periods of up to 10 days, a lot of the ribonucleic acid (RNA) is still intact and cells may still be cultured. The use of chilling and other methods to reduce injury and normalise explants have not been investigated.

If the mechanical environment is not recapitulated, is cell culture relevant?

Tenocytes seem to have a mechanostat set point (i.e. an ideal level of strain depending on the tendon of origin), but this does alter with strain deprivation over a certain period of time [50]. The tenocytes have cilia, the deflection and length of which is modulated by tensile loading [51]. These have not been studied in horses but might be of use to establish the normality of *in vitro* strain environments. The zero-strain environment that most cells experience in culture is not desirable, because tendon cells express matrix metalloproteinases in that state [47,52]. The commercially available Flexcell system is one means of applying static or cyclical strain, either to monolayers on deformable membranes (with a recognised 'edge effect') or bioartificial tendons. Other methods have been used to test both bioartificial tendons and explants/fascicles, including Bose system adaptations or custom-made systems. Technical issues can include the following: effects and efficacy of gripping the ends; keeping specimens moist (with fluid affecting optical measurements); rupture due to cell-induced rather than externally applied tension; and visualisation of cells under strain (this is possible under slow or incremental strain) [53]. The effects of mechanical strain on tendon cells are well known, and the cells in bioartificial tendons have been shown to become more 'tenocyte like' with application of cyclical strain [43]. However, there is still a problem when determining which strain environments stimulate optimal tenocyte differentiation or stress, i.e. this may not correlate with responses that would occur *in vivo*.

Important research themes

The following themes were considered to be some of the most important directions for research into the prevention of SDFT injury in equine athletes in the immediate future.

- Track-side and horse-side:
  - Large-scale studies appropriately staffed by researchers (this would presumably need to be multi-institutional) to identify associations between multiple factors and SDFT injury occurrence (e.g. track surface conditions, weather, specific training regimens and individual horse factors), including the use of GPS systems and other monitors.
  - Investigation of early exercise (and mental attitude) of foals relative to subsequent performance, i.e. interacting with stud farms.
  - Further development of imaging strategies for detection of early pathology in conjunction with medical colleagues and including, but not limited to, ultrasound tissue characterisation, magnetic resonance imaging and power Doppler techniques.
  - Consideration of using other training environments that might be more practical, e.g. eventing yards.
- Horse-side and in the laboratory:
  - Examination of large numbers of SDFT samples from horses of known training/racing history to identify early pathology (again this would need to be multi-institutional and include multiple techniques).
  - Development of multiplex tests to identify blood-based phenotypic markers of early injury (to be related to the tissue study above) prior to field studies. This could potentially include modelling of horses most at risk in order to focus the sampling.
  - Measurement of the SDFT environment during exercise, including oxygenation (oximetry), temperature and cellular activity (e.g. by paratenon dialysis, positron emission tomography scanning) for application to cell culture models.
  - Development of a panel of cell stress markers for the horse, and determination of the effects of multiple factors, including those measured *in vivo* (above), cell culture stress, matrix type and nature and injury status of the original tissue.
  - Refinement of cell culture models, including study of paratenon and epitenon cells separately, further investigation of the acellular matrix method, validation of 2D data in 3D systems, improvement of explant viability and application of cyclical strain.

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References

1. Avella, C.S., Price, J.S., Smith, R.K., Wood, J.L. and Verheyen, K.L. (2009) Descriptive epidemiology of fracture, tendon and suspensory ligament injuries in National Hunt racehorses in training. *Equine Vet. J.* **41**, 372-378.
2. Lam, K.H., Parkin, T.D., Riggs, C.M. and Morgan, K.L. (2007) Descriptive analysis of retirement of Thoroughbred racehorses due to tendon injuries at the Hong Kong Jockey Club (1992-2004). *Equine Vet. J.* **39**, 143-148.
3. Setterbo, J.J., Fyhrie, P.B., Hubbard, M., Upadhyaya, S.K. and Stover, M.M. (2013) Dynamic properties of a dirt and synthetic equine racetrack surface measured by a track-testing device. *Equine Vet. J.* **45**, 25-30.
4. Williams, R.B., Harkins, L.S., Hammond, C.J. and Wood, J.L. (2001) Racehorse injuries, clinical problems and fatalities recorded on British racecourses from flat racing and National Hunt racing during 1996, 1997 and 1998. *Equine Vet. J.* **33**, 478-486.
5. Reardon, R.J.M., Boden, L.A., Mellor, D.J., Love, S., Newton, J.R., Stirk, A.J. and Parkin, T.D.H. (2013) Risk factors for superficial digital flexor tendinopathy in Thoroughbred racehorses in steeplechase starts in the United Kingdom (2001-2009). *Vet. J.* **195**, 325-330.
6. Peterson, M.L., Reiser, R.F., Kuo, P.H., Radford, D.W. and McIlwraith, C.W. (2010) Effect of temperature on race times on a synthetic surface. *Equine Vet. J.* **42**, 351-357.
7. Wilson, A.M. and Goodship, A.E. (1994) Exercise-induced hyperthermia as a possible mechanism for tendon degeneration. *J. Biomech.* **27**, 899-905.
8. Weller, R., Pfau, T., Verheyen, K., May, S.A. and Wilson, A.M. (2006) The effect of conformation on orthopaedic health and performance in a cohort of National Hunt racehorses: preliminary results. *Equine Vet. J.* **38**, 622-627.
9. Kasashima, Y., Smith, R.K., Birch, H.L., Takahashi, T., Kusano, K. and Goodship, A.E. (2002) Exercise-induced tendon hypertrophy: cross-sectional area changes during growth are influenced by exercise. *Equine Vet. J., Suppl.* **34**, 264-268.
10. Moffat, P.A., Firth, E.C., Rogers, C.W., Smith, R.K., Barneveld, A., Goodship, A.E., Kawcak, C.E., McIlwraith, C.W. and Van Weeren, P.R. (2008) The influence of exercise during growth on ultrasonographic parameters of the superficial digital flexor tendon of young Thoroughbred horses. *Equine Vet. J.* **40**, 136-140.
11. Rogers, C.W., Firth, E.C., McIlwraith, C.W., Barneveld, A., Goodship, A.E., Kawcak, C.E., Smith, R.K. and Van Weeren, P.R. (2008) Evaluation of a new strategy to modulate skeletal development in racehorses during growth: the effects on 2- and 3-year-old racing careers. *Equine Vet. J.* **40**, 119-127.
12. Tjønna, A.E., Leinan, I.M., Bartnes, A.T., Jenssen, B.M., Gibala, M.J., Winett, R.A. and Wisløff, U. (2013) Low- and high-volume of intensive endurance training significantly improves maximal oxygen uptake after 10-weeks of training in healthy men. *PLoS ONE* **8**, e65382.
13. Parkin, T.D.H. (2008) Epidemiology of racetrack injuries in racehorses. *Vet. Clin. N. Am.: Equine Pract.* **24**, 1-19.
14. Scott, A., Docking, S., Vincenzino, B., Alfredson, H., Murphy, R.J., Carr, A.J., Zwerver, J., Lundgreen, K., Finlay, O., Pollock, N., Cook, J.L., Fearon, N., Purdam, C.R., Hoens, A., Rees, J.D., Goetz, T.J. and Danielson, P. (2013) Sports and exercise-related tendinopathies: a review of selected topical issues by the participants of the second International Scientific Tendinopathy Symposium (ISTS) Vancouver 2012. *Br. J. Sports Med.* **47**, 536-544.
15. Heard, B.J., Fritzler, M.J., Wiley, J.P., McAllister, J., Martin, L., El-Gabalawy, H., Hart, D.A., Frank, C.B. and Krawertz, R. (2013) Intraarticular and systemic inflammatory profiles may identify patients with osteoarthritis. *J. Rheumatol.* **40**, 1379-1387.
16. Thorpe, C.T., Udeze, C.P., Birch, H.L., Clegg, P.D. and Screen, H.R. (2012) Specialization of tendon mechanical properties results from interfascicular differences. *J. R. Soc. Interface* **9**, 3108-3117.
17. Longo, U.G., Oliva, F., Denaro, V. and Maffulli, N. (2008) Oxygen species and overuse tendinopathy in athletes. *Disabil. Rehabil.* **30**, 1563-1571.
18. Millar, N.L., Reilly, J.H., Kerr, S.C., Campbell, A.L., Little, K.J., Leach, W.J., Rooney, B.P., Murrell, G.A. and McInnes, I.B. (2012) Hypoxia: a critical regulator of early human tendinopathy. *Ann. Rheum. Dis.* **71**, 302-310.
19. Birch, H.L., Bailey, A.J. and Goodship, A.E. (1998) Macroscopic 'degeneration' of equine superficial digital flexor tendon is accompanied by a change in extracellular matrix composition. *Equine Vet. J.* **30**, 534-539.
20. Dyment, N.A., Liu, C.F., Kazemi, N., Aschbacher-Smith, L.E., Kenter, K., Breidenbach, A.P., Shearn, J.T., Wylie, C., Rowe, D.W. and Butler, D.L. (2013) The paratenon contributes to scleraxis-expressing cells during tendon healing. *PLoS ONE* **8**, e59944.
21. Heinemeier, K.M., Schjerling, P., Heinemeier, J., Magnusson, S.P. and Kjaer, M. (2013) Lack of tissue renewal in human adult Achilles tendon is revealed by nuclear bomb ^{14}C. *FASEB J.* **27**, 2074-2079.
22. Thorpe, C.T., Streeter, I., Pinchbeck, G.L., Goodship, A.E., Clegg, P.D. and Birch, H.L. (2010) Aspartic acid racemization and collagen degradation markers reveal an accumulation of damage in tendon collagen that is enhanced with aging. *J. Biol. Chem.* **285**, 15674-15681.
23. Birch, H.L., Wilson, A.M. and Goodship, A.E. (2008) Physical activity: does long-term, high-intensity exercise in horses result in tendon degeneration? *J. Appl. Physiol.* **105**, 1927-1933.
24. Thorpe, C.T., Birch, H.L., Clegg, P.D. and Screen, H.R.C. (2013) The role of the non-collagenous matrix in tendon function. *Int. J. Exp. Pathol.* **94**, 248-259.
25. Kohrs, R.T., Zhao, C., Sun, Y.L., Jay, G.D., Zhang, L., Warman, M.L., An, K.N. and Amadio, P.C. (2011) Tendon fascicle gliding in wild type heterozygous, and lubricin knockout mice. *J. Orthop. Res.* **29**, 384-389.
26. Van Schie, H.T.M., Docking, S.I., Daffy, J., Praet, S.E., Rosengarten, S. and Cook, J.L. (2013) Ultrasound tissue characterization, an innovative technique for injury-prevention and monitoring of tendinopathy. *Br. J. Sports Med.* **47**, e2.

27. Docking, S.I., Daffy, J., van Schie, H.T. and Cook, J.L. (2012) Tendon structure changes after maximal exercise in the Thoroughbred horse: use of ultrasound tissue characterisation to detect in vivo tendon response. *Vet. J.* **194**, 338-342.
28. Moerch, L., Pingel, J., Boesen, M., Kjaer, M. and Langberg, H. (2013) The effect of acute exercise on collagen turnover in human tendons: influence of prior immobilization period. *Eur. J. Appl. Physiol.* **113**, 449-455.
29. Wang, P.H., Luh, J.J., Chen, W.S. and Li, M.L. (2011) *In vivo* photoacoustic micro-imaging of microvascular changes for Achilles tendon injury on a mouse model. *Biomed. Opt. Express.* **2**, 1462-1469.
30. Mordant, D.J., Al-Abboud, I., Muyo, G., Gorman, A., Sallam, A., Ritchie, P., Harvey, A.R. and McNaught, A.I. (2011) Spectral imaging of the retina. *Eye (Lond)* **25**, 309-320.
31. McNaught, A., Harvey, A., Muyo, G., Mordant, D., Al-Abboud, I. and Ritchie, P. (2009) Retinal vessel oximetry using sequently and 'snapshot' hyperspectral imaging. *Acta Ophthalmol.* **87**, s244.
32. Notamicola, A., Fischetti, F., Gallone, D., Moretti, L., Pignataro, P., Tafuri, S. and Moretti, B. (2012) Overload and neovascularization of shoulder tendons in volleyball players. *BMC Res. Notes* **5**, 397.
33. Langberg, H., Rosendal, L. and Kjaer, M. (2001) Training-induced changes in peritendinous type I collagen turnover determined by microdialysis in humans. *J. Physiol.* **534**, 297-302.
34. Smith, R.K. and Heinegård, D. (2000) Cartilage oligomeric matrix protein (COMP) levels in digital sheath synovial fluid and serum with tendon injury. *Equine Vet. J.* **32**, 52-58.
35. Tully, L.J., Murphy, A.M., Smith, R.K.W., Hulin-Curtis, S.L., Verheyen, K.L.P. and Price, J.S. (2014) Polymorphisms in *TNC* and *COL5A1* genes are associated with risk of superficial digital flexor tendinopathy in National Hunt Thoroughbred racehorses. *Equine Vet. J.* 46, 289-293.
36. Zhang, J. and Wang, J.H. (2010) Characterization of differential properties of rabbit tendon stem cells and tenocytes. *BMC Musculoskelet. Disord.* **11**, 10.
37. Harris, Q., Seto, J., O'Brien, K., Lee, P.S., Kondo, C., Heard, B.J., Hart, D.A. and Krawertz, R.J. (2013) Monocyte chemotactic protein-1 inhibits chondrogenesis of synovial mesenchymal progenitor cells: an in vitro study. *Stem Cells* **31**, 2253-2265.
38. Ando, W., Heard, B.J., Chung, M., Nakamura, N., Frank, C.B. and Hart, D.A. (2012) Ovine synovial membrane-derived mesenchymal progenitor cells retain the phenotype of the original tissue that was exposed to in-vivo inflammation: evidence for a suppressed chondrogenic differentiation potential of the cells. *Inflamm. Res.* **61**, 599-608.
39. Wright, W.E. and Shay, J.W. (2006) Inexpensive low-oxygen incubators. *Nat. Protoc.* **1**, 2088-2090.
40. Rich, T., Henderson, L., Becker, D.L., Cornell, H. and Patterson-Kane, J.C. (2013) Indicators of replicative damage in equine tendon fibroblast monolayers. *BMC Vet. Res.* **9**, 180.
41. Cook, J.L., Feller, J.A., Bonar, S.F. and Khan, K.M. (2004) Abnormal tenocyte morphology is more prevalent than collagen disruption in asymptomatic athletes' patellar tendons. *J. Orthop. Res.* **22**, 334-338.
42. Richardson, L., Dudhia, J., Sadler, C.J., Goodship, A.E. and Smith, R.K. (2007) Extracellular matrix cues for mesenchymal stem cell differentiation. *Proc. 53rd Orthop. Res. Soc.* **32**, 472.
43. Scott, A., Danielson, P., Abraham, T., Fong, G., Sampaio, A.V. and Underhill, T.M. (2011) Mechanical force modulates scleraxis expression in bioartificial tendons. *J. Musculoskelet. Neuronal Interact.* **11**, 124-132.
44. Kapacee, Z., Richardson, S.H., Lu, Y., Starborg, T., Holmes, D.F., Baar, K. and Kadler, K. (2008) Tension is required for fibropositor formation. *Matrix Biol.* **27**, 371-375.
45. Stanley, R.L., Fleck, R.A., Becker, D.L., Goodship, A.E., Ralphs, J.R. and Patterson-Kane, J.C. (2007) Gap junction protein expression and cellularity: comparison of immature and adult equine digital tendons. *J. Anat.* **211**, 325-334.
46. Young, N.J., Becker, D.L., Fleck, R.A., Goodship, A.E. and Patterson-Kane, J.C. (2009) Maturational alterations in gap junction expression and associated collagen synthesis in response to tendon function. *Matrix Biol.* **28**, 311-323.
47. Dudhia, J., Scott, C.M., Draper, E.R.C., Heinegård, D., Pitsillides, A.A. and Smith, R.K. (2007) Aging enhances a mechanically-induced reduction in tendon strength by an active process involving matrix metalloproteinase activity. *Aging Cell.* **6**, 547-566.
48. Dakin, S.G., Dudhia, J., Werling, N.J., Werling, D., Abayasekara, D.R.E. and Smith, R.K.W. (2012) Inflamm-aging and arachadonic acid metabolite differences with stage of tendon disease. *PLoS ONE* **7**, e48978.
49. Dakin, S.G., Werling, D., Hibbert, A., Abayasekara, D.R.E., Young, N.J., Smith, R.K.W. and Dudhia, J. (2012) Macrophage sub-populations and the lipoxin A_4 receptor implicate active inflammation during equine tendon repair. *PLoS ONE* **7**, e32333.
50. Arnoczky, S.P., Lavagnino, M., Egerbacher, M., Caballero, O., Gardner, K. and Shender, M.A. (2008) Loss of homeostatic strain alters mechostat 'set point' of tendon cells. *Clin. Orthop. Relat. Res.* **466**, 1583-1591.
51. Lavagnino, M., Gardner, K., Sediak, A.M. and Arnoczky, S.P. (2013) Tendon cell ciliary length as a biomarker of *in situ* cytoskeletal tensional homeostasis. *Muscles Ligaments Tendons J.* **3**, 118-121.
52. Gardner, K., Arnoczky, S.P., Caballero, O. and Lavagnino, M. (2008) The effect of stress-deprivation and cyclic loading on the TIMP/MMP ratio in tendon cells: an *in vitro* experimental study. *Disabil. Rehabil.* **30**, 1523-1529.
53. Screen, H.R., Lee, D.A., Bader, D.L. and Shelton, J.C. (2004) Development of a technique to determine strains in tendons using the cell nuclei. *Biorheology* **40**, 361-368.

Supporting information

Additional Supporting Information may be found in the online version of this article at the publisher's website:

Supplementary Item: Workshop participants.