Exposure of tendon extracellular matrix to synovial fluid triggers endogenous and engrafted cell death: A mechanism for failed healing of intrathecal tendon injuries

E.R. Garvican, M. Salavati, R.K.W. Smith*, J. Dudhia

Clinical Sciences and Services, The Royal Veterinary College, Hawkshead Lane, North Mymms, Hertfordshire, AL9 7TA

*Corresponding author: rksmith@rvc.ac.uk

Running title: *Synovial fluid causes death of tenocytes*

Abstract

Aim: The purpose of this study was to investigate the effect of normal synovial fluid (SF) on exposed endogenous tendon-derived cells (TDC) and engrafted mesenchymal stem cells (MSCs) within the tendon extracellular matrix.

Methods: Explants from equine superficial digital flexor (extra-synovial) and deep digital flexor tendons (DDFT) from the compressed, intra-synovial and the tensile, extra-synovial regions were cultured in allogeneic or autologous SF-media. Human hamstring explants were cultured in allogeneic SF. Explant viability was assessed by staining. Proliferation of equine monolayer MSCs and TDCs in SF-media and co-culture with DDFT explants was determined by alamarblue®. Non-viable Native Tendon matrices (NNTs) were re-populated with MSCs or TDCs and cultured in SF-media. Immunohistochemical staining of tendon sections for the apoptotic proteins caspase-3, -8 and -9 was performed.

Results: Contact with autologous or allogeneic SF resulted in rapid death of resident tenocytes in equine and human tendon. SF did not affect the viability of equine epitenon

cells, or of MSCs and TDCs in monolayer or indirect explant co-culture. MSCs and TDCs, engrafted into NNTs, died when cultured in SF. Caspase-3, -8 and -9 expression was greatest in SDFT explants exposed to allogeneic SF.

Conclusions: The efficacy of cells administered intra-synovially for tendon lesion repair is likely to be limited, since once incorporated into the matrix, cells become vulnerable to the adverse effects of SF. These observations could account for the poor success rate of intra-synovial tendon healing following damage to the epitenon and contact with SF, common with most soft tissue intra-synovial pathologies.

Keywords*:* tendon, synovial fluid, viability, mesenchymal stem cell, extracellular matrix

Received 16 March 2016; Revised 26 September 2016; Accepted 03 October 2016

Introduction

Rotator cuff disease is the third most common orthopaedic complaint in general practice [1, 2]. It is estimated that rotator cuff tears affect 5-30% of adults (rising to approximately 60% of over 60 year olds) and that 40-50% of patients in the UK consult their general practitioners for shoulder pain [3, 4]. Despite this high prevalence, both medical and surgical treatment modalities are far from universally successful, with re-tear rates as high as 89% in tears classified as large or massive [5-7]. Effective repair of injured tissue along with restoration of biomechanical properties and cellular organisation is not consistently achieved. Even with the use of modern surgical techniques which attempt to bridge ruptured tendon gaps by means of grafts coupled with lengthy, costly rehabilitation, 40–50% of patients continue to have symptoms and/or considerable functional impairment 1 – 2 years post-diagnosis [8-10]. Therefore there remains a strong need to address the reasons for these limitations on successful tendon repair.

There are no good laboratory models of human tendon disease [11], in part due to the anatomical differences between bipeds and quadrupeds. However, there are striking similarities between the horse and human when comparing functionally-similar tendons. Thus the equine deep digital flexor tendon (DDFT) in the distal forelimb frequently suffers naturally-occurring intra-synovial tears comparable to rotator cuff tears. Both tendons are located in a similar biomechanical environment (that of compression), in an intra-synovial location, suffer age-related degeneration and demonstrate failed healing with persistent pain. Surgical debridement in isolation does not generate acceptable success rates [12] with 60-62% of horses diagnosed with DDFT tears unable to return to their previous level of work and the prognosis worsening to 82% for larger tears [13].

The intra-synovial location of both these tendons provides different treatment challenges to that of extra-synovial tendon injury. Release of extracellular matrix (ECM) proteins [14], cytokines [15, 16], and matrix metalloproteinases [17] from the exposed damaged tendon surface are important drivers for persistent synovial inflammation. Together with a sustained compressive environment, where a tendon sheath or bursa exists to ease movement of the tendon over a bony prominence, these factors limit natural tendon healing.

Mesenchymal stem cells (MSCs) have been defined by their functional capacity to self-renew and to generate a range of differentiated progeny [18]. Enhancing natural tendon repair using autologous bone marrow-derived MSCs is an attractive concept, which is supported by positive results in both clinical and experimental studies in the horse but to date these have been limited to extra-synovial tendon injuries [19, 20]. Little is currently known about the effect of synovial fluid on implanted cells. Studies have shown suppression of growth rates of human chondrocytes [21] and cruciate ligament fibroblasts [22] when using SF with no additional nutrient supplementation; when SF was used in combination with cell-specific growth medium, chondrocyte culture was deemed feasible [23]. Previous studies have

reported that synovial fluid has a deleterious effect on tendon-to-bone healing in an *in vivo* rabbit model [24] but this study measured histological and biomechanical parameters, not tissue viability. Clinical observation demonstrates that fluid leakage from a damaged synovial cavity results in significant reaction and inflammation of the tissues exposed to the synovial fluid, supporting the hypothesis that synovial fluid has adverse effects on tissues not normally exposed to it.

We have previously demonstrated unaffected adherence of MSCs and tendon-dervied cells (TDCs) in synovial fluid suspension to the matrix of tendon explants [25] but our observations during that study led us to further investigate the gross effect of normal synovial fluid on the cells resident in exposed tendon tissue as well as those which might be implanted following injury.

Materials & Methods:

Collection of tendon: Equine tendons were collected at postmortem under approval from the Ethics and Welfare Committee at the Royal Veterinary College (URN 2011–1117). No horses were euthanized for the sole purpose of obtaining tissues for this study. Macroscopically normal metacarpal tensile region SDFTs as well as DDFTs from both the metacarpophalangeal compressed (C-DDFT) and metacarpal tensile (T-DDFT) regions were aseptically harvested from three skeletally mature horses (9 ± 3 years), without orthopaedic disease. The tendons were kept in complete medium (D10; DMEM, supplemented with 10% v/v fetal bovine serum, 100 U/mL penicillin, and 100 U/mL streptomycin (all from Invitrogen, Paisley, UK)) until explant dissection. Explants were prepared using a cutting template, which held three cryoblades [26], such that uniform pieces measuring 2 × 0.20 × 0.20 cm could be cut. Explants were composed of internal (core) tendon tissue and cut along the longitudinal axis of the tendon in order to expose an internal surface. In addition, larger

explants which included an intact epitenon on one side were prepared by sharp dissection. Tendon explants were maintained in D10 in 12-well culture plates (Starlab, Milton Keynes, UK) for 24 h prior to experimentation. All culture incubations of tendon and cells, were performed under standard culture conditions (37°C in a humidified atmosphere of 5% CO_2).

Human hamstring tendon was supplied by the Oxford Musculoskeletal Biobank (OMB) following approval from the Ethics and Welfare Committee at the Royal Veterinary College (reference number 2015 0128H) and OMB Access and Development Committee (Oxford REC C09/H0606/11). Samples of normal hamstring were obtained from two patients (1 male, 25 yrs old and 1 female, 27 years old) undergoing cruciate ligament reconstruction surgery but otherwise deemed healthy. Further personal or identifying information was not collected. Tissue deemed to be surplus was collected at the culmination of surgery. Explants were cut with a scalpel blade to measure approximately 2 x 0.5 x 0.2 cm.

Isolation of cells: Equine bone marrow MSC populations were obtained from surplus cells, used therapeutically to treat SDFT lesions in cases referred to our clinic. TDCs were obtained from samples of SDFT which were finely diced and digested with pronase (1% w/v in D10) for 1 h, then collagenase (0.5% w/v in D10; Worthingtons, UK) for 18 h. Cells were recovered following straining of the digest through a 70 μm filter and centrifugation, and seeded in culture flasks at 5,000 cells/cm^2. Passage 0–Passage 2 (P0–P2) cells were re-suspended in Cellbanker cell freezing medium (Amsbio, Oxfordshire, UK) and stored in liquid nitrogen until use.

Collection of synovial fluid: Synovial fluid was aseptically aspirated from the carpal joints (to obtain adequate volume) of the same horses from which gross tendon samples were obtained and immediately centrifuged (at 500 *g* for 15 min) to pellet any cellular component. The supernatant was heated at 60°C for 1 h with regular mixing by inversion to denature complement and stored at -20°C prior to use. For experiments, powdered DMEM (Sigma-

Aldrich, Poole, UK) was reconstituted with equine synovial fluid, to the manufacturer's recommended concentration and supplemented with 10% v/v fetal calf serum, 100 U/mL penicillin and 100 U/mL streptomycin (as before). The resultant synovial fluid media (SF-media) was pH 7.8. For experiments utilising allogeneic SF, samples were pooled to provide adequate volume.

Synovial fluid was also aspirated from the femoropatellar joints of two human patients (one male, aged 69 years and one female aged 76 years) undergoing total knee replacement surgery, and treated as above. Human SF was not reconstituted with powdered media, since prior experimentation with equine SF had shown cell death to be so rapid, nutrition was not considered a factor.

Culture of tendon in synovial fluid: Equine tendon explants were cultured in 2 mL D10 or SF-media made from autologous SF or allogeneic SF. Paired explants from each horse were removed after 30 min, 1, 6 and 24 h and processed for cell viability staining or immunohistochemistry. Human tendon explants were cultured in 3mL of D20 (DMEM, supplemented as before, but with 20% v/v fetal bovine serum) or SF for 1 h prior to viability staining.

Proliferation of cells in monolayer: To determine the effect of SF-media on cell proliferation, 20,000 equine MSCs (n=3) and TDCs (n=3) were seeded into wells of a 12 well culture plate (Starlab, Milton Keynes, UK) and allowed to adhere overnight. The following day media was replaced with 1 mL 10% alamarBlue® reagent (AbD Serotec, Oxfordshire, UK) in D10. Cells were incubated for a further 4 h, shielded from light. 100 µL aliquots of media were then transferred, in triplicate, to fluoro-microtitre plates (SPL LifeSciences, UK) and fluorescence was measured at 570 nm (excitation) and 585 nm (emission) (Infinite M200

PRO fluorometer, Tecan, UK). Media was replaced with 2 mL fresh D10 or SF-media. The assay was repeated at 2 and 6 days.

Co-culture of explants and cells: Equine MSCs and TDCs (10 x 10^6 cells, n=3 for each cell type) were seeded into each well of a 6-well culture dish (BD Biosciences, Durham, NC, USA) and allowed to adhere overnight. The next day, transwell inserts (VWR, Leicester, UK) were inserted into each well and the upper and lower chambers were filled with SF-media. Live C-DDFT explants (one donor per well, n = 3 horses) were then placed into the transwell for 1, 4 or 24 h, after which the explant cell viability was assessed. The remaining monolayer was washed 3 times with PBS and cell metabolic activity was assessed using the alamarBlue® assay as previously described.

Non-viable Native Tendon Matrices (NNTs): Additional equine DDFT (n = 3) explants were subjected to repeated (minimum 3 cycles) freeze-thaw cycles (-80 °C snap-freeze for > 2 h, followed by rapid thaw at 37 °C for 30 min) in order to render all resident tenocytes non-viable (as assessed by viability staining (data not shown)) [26]. Subsequently, MSCs or TDCs (1 x 10^6 cells, n =3) were co-cultured overnight with NNTs (4 NNTs per well, 2mL D10 per well). Following adherence of cells [25] NNTs were cultured for 3 weeks to allow migration of cells into the matrix [25]. Re-cellularised NNTs were then incubated with allogeneic SF-media or fresh D10 for 1 h and cell viability assessed.

Viability staining: Explants were incubated in phosphate-buffered saline (PBS (Sigma, Poole, UK)) containing 2 μM Calcein AM and 4 μM propidium iodide dyes (both from Invitrogen, Paisley, UK) for 1 h and then visualized under a fluorescent microscope (Leica, UK) to determine the ratio of viable (emission λ 520 nm) to dead (emission λ 610 nm) cells. The percentage viability was calculated from an average of 3 fields.

Immunohistochemistry: Equine tendon explants (n=3 horses) were fixed in PBS-buffered formalin 10% (Sigma, Poole, UK) immediately upon termination of the culture for minimum of 2 days. After 48 h, explants were transferred to 100% ethanol (Merck Millipore, UK) for 4 h and maintained in 70% ethanol. Explants were then processed by dehydration and embedded in wax and 8 mm sections were cut and mounted on Super frost slides (Fisher Scientific, UK). Sections were probed using equine specific antibodies (Caspase 9 Antibody Rabbit pAb (biorbyt, UK); Caspase 3 Antibody Rabbit pAb (LS Bio, UK); Caspase 8 antibody (N1C1) Rabbit Poly Clonal (Genetex, UK)) all at a final concentration of 1:500 using the EXPOSE Mouse and Rabbit Specific HRP/DAB Detection IHC kit (Abcam, UK) according to the manufacturer's instructions. Briefly, slides were rehydrated using decreasing concentrations of ethanol (100%, 90%, 70% and 50% for 2 min each). After blocking the endogenous peroxidase activity using EXPOSE protein block (Abcam, UK), primary antibodies were diluted in PBS containing 0.05%Tween 20 (Sigma, Poole, UK) and placed on slides (150 µL/ section) overnight at 4ºC. Secondary antibody and DAB development were carried out according to the manufacturer's instructions. Nuclear counterstain with Haematoxylin (15 sec/slide) was conducted prior to mounting the slide using DPX mounting media (Sigma, Poole, UK) and 20 mm x 20 mm coverslips.

Densitometry and analysis of IHC slides: Multiple images of regions of interest (at least 4) were taken from each using a camera mounted Leica microscope (Leica, UK; Image ProPlus software v.5). Images were analysed using Image J software via Colour Deconvolution plugin and integrated density value (IDV) area fraction measurement [27]. Area fraction containing the DAB signal was also normalized against the total cell count (using the Haematoxylin density) in each image and adjusted for percentage viability.

Results

Approximately 95% of epitenon cells and 50% of tenocytes resident in explants cultured in D10 (control explants) remained viable throughout 24 h of the experiment (Figure 1). This is similar to previously reported explant viability [28]. Cells within the epitenon exposed to SF remained viable (>95% viability after 24 h). In contrast, the viability of SDFT, C-DDFT and T-DDFT explants exposed to SF- media decreased rapidly (Figures 1 and 2) such that within 30 min, explant viability was less than 10%. After 1 h no living cells could be detected in explants exposed to SF media. There was no significant difference between allogeneic or autologous SF in their effects on cell viability (Figure 2). As a result, all future experiments utilised allogeneic SF, due to ease of collection. This experiment was repeated >20 times with a variety of normal equine DDFT exposed to autologous SF and resulted each time in rapid death of all tendon cells (data not shown).

Cells within human hamstring remained viable when cultured in D20 (>90% viability at 6 h post surgery). Human hamstring explants exposed to allogeneic SF for 1 h contained no living tenocytes, however the cells of the vasculature within the tendon ECM remained viable in sections of hamstring exposed to SF (Figure 3).

In contrast to the findings in tendon explants, MSCs and TDCs cultured in SF-media in monolayer, on tissue culture plastic, remained viable (Figure 4a, b). In order to determine if the exposure of the tendon ECM to SF resulted in the release of a cytotoxic signal, monolayer cells were co-cultured with explants. There was no difference in metabolic activity when MSC and TDC monolayers co-cultured with C-DDFT explants were exposed to SF or to D10 (Figure 4 c, d) for up to 24 h. However, the tenocytes resident within C-DDFT explants died rapidly on exposure to SF as before.

When NNTs re-cellularised with equine MSCs or TDCs were cultured in SF-media for 1 h, all cells adhered to the matrix died. In contrast, when re-cellularised NNTs were cultured in D10, these cells remained viable (Figure 5).

Baseline levels of all three caspases were expressed in control tendon explants cultured in D10 and in tendon explants cultured in both autologous and allogeneic SF. Densitometric analysis standardised to the percentage of live cells observed within the explants, showed that expression was increased in all explants exposed to autologous or allogeneic SF media and was greatest in SDFT explants exposed to allogeneic SF media (Figure 6).

Discussion

Our experiments have demonstrated that the viability of cells of the synovial lining surrounding the tendon (epitenon) are unaffected by synovial fluid, with which it is constantly in contact and from which it derives nutrition [29]. However, exposure of the tendon core to normal SF, which can happen once the epitenon is damaged, results in the rapid death of the native resident cells or those adhered to the exposed matrix. This finding may explain why damage to intra-synovial tendons carries such a poor clinical prognosis for both the horse and human as the viability of reparative cells is compromised in the injury.

Death of tenocytes resident within the matrix was extremely rapid. However, the death of cells did not result in the release of a diffusible cytotoxic signal (evidenced by successful indirect co-culture of a monolayer) indicating that the causal mechanism requires physical contact between cells and the ECM. Whilst the non-cellular, extracellular matrix of tendon was thought of as a relatively inactive component of tendon, it is now recognised to play important roles in energy storage and function [30] and so may also orchestrate apoptotic signals. However, such rapid death of resident tenocytes supports a relative lack of production of the apoptotic protein cascade as this would require continuing metabolic

activity within the condemned cell. As a result, it was not possible to determine whether the cell itself initiates apoptosis (intrinsic cell death) or whether this occurs through an external stimulus (extrinsic cell death) [31].

In contrast, both MSCs and TDCs can be successfully cultured in monolayer in media composed of synovial fluid, albeit with a reduced rate of proliferation similar to that reported with other cell types [21, 22]. The use of SF-media in the current report allows the exclusion of nutrient deficiency or complement activation as causal factors, but the reasons for suppression of cell proliferation are unknown. The components of synovial fluid require further investigation in order to determine the constituents responsible for this effect and the mechanism of their interaction with the ECM. It is intriguing that the cells of the vasculature and the epitenon are not affected by the SF. We assume that the cells within these tissues either have a mechanism to protect from the SF or that they also may be susceptible to its effect if implanted within the tendon ECM, although we have not investigated this in the current work. Our observations that tendon explants co-cultured with cell monolayers die, whilst the monolayer remains viable indicates there was no release of cytotoxic paracrine factor from the tendon explant nor was there a protective effect of a co-cultured monolayer on tendon tissue.

As we have previously demonstrated, MSCs (derived from bone marrow, or synovial fluid) suspended in either normal or inflammatory synovial fluid will adhere to the cut surfaces of a tendon explant [25] indicating no diminished viability or adherence capacity under these conditions. Throughout tissue culture, surface adherent cells migrate into the matrix to a depth of at least 500μm by 21 days and align in the direction of the resident collagen fibres and expressing an elongated morphology similar to resident tenocytes [25]. However, once these cells become resident within the matrix, they become susceptible to the lethal effects of synovial fluid in a similar way to resident tenocytes. This is of significance when considering

the use of MSCs for therapy of intra-synovial tendon tears, since although implanted MSCs may adhere to the site of tendon injury, engrafted MSCs will subsequently die *in situ*. Future therapies aimed at intra-synovial tendon healing should incorporate a physical repair to the epitenon, protecting the tendon ECM from contact with synovial fluid, beneath which MSCs can be implanted.

The human rotator cuff and the equine DDFT share many functional and anatomical properties, suffer injuries caused by very similar underlying pathologies and suffer a high rate of treatment failure. Human hamstring tendon is also a flexor tendon like the horse DDFT and human rotator cuff tendons. Its use during reconstruction of the internal knee ligaments in cases of anterior cruciate ligament rupture meant that excess tissue was available for these experiments. It has the advantage of being normal tendon and, although it is normally an extrasynovial tendon, it is used intrasynovially during ACL reconstruction. ACL injuries commonly occur in the 30 – 40 year group which is equivalent to the age range of horses used. Therefore, the results of this study are highly relevant for the very common ACL repair technique and further supports the horse as an appropriate model for human disease. Damage to an intra-synovial tendon, such as the human rotator cuff or the equine deep digital flexor tendon causing exposure of core tendon tissue to the cytotoxic effects of synovial fluid, will result in death of resident tenocytes. This may explain the poor success rate of current treatment modalities and future therapy should investigate methods by which the tendon defect can be sealed. Without a physical barrier separating synovial fluid from implanted MSCs, cell therapy is unlikely to deliver effective healing.

Sources of funding:

This work was funded by the Medical Research Council UK (MRC GO902406) and the Biotechnology and Biotechnology and Biological Sciences Research Council UK (BBSRC BB/J000655/1).

Declaration of Interest:

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

Acknowledgments:

The authors wish to thank the staff at the Oxford Musculoskeletal Biobank for their assistance with sample collection.

References

1. Stevenson JH, Trojian T. Evaluation of shoulder pain. J Family Prac. 2002;51(7):605-611.
2. McCormick A, Fleming D, Charlton J. Morbidity statistics from general practice. Fourth national study 1991–92. OPCS RD, London 1996; HMSO 55.
3. Badcock LJ, Lewis M, Hay EM, Croft PR. Consultation and the outcome of shoulder-neck pain: a cohort study in the population. J Rheumatol 2003;30:2694-2699.
4. Walker Bone K, Palmer KT, Reading I, Coggon D, Cooper C. Prevalence and impact of musculoskeletal disorders of the upper limb in the general population. Arthritis & Rheum. 2004;51:642-651.
5. Galatz LM, Ball CM, Teefey SA, Middleton WD, Yamaguchi K. The outcome and repair integrity of completely arthroscopically repaired large and massive rotator cuff tears. J Bone Joint Surg Am. 2004;86-A(2):219-224.
6. Gazielly DF, Gleyze P, Montagnon C. Functional and anatomical results after rotator cuff repair. Clin Orthop Relat Res. 1994;304:43-53.
7. Harryman DT, Mack LA, Wang KY, Jackins SE, Richardson ML, Matsen FA. Repairs of the rotator cuff. Correlation of functional results with integrity of the cuff. J Bone Joint Surg Am. 1991;73:982-989.
8. van der Windt DA, Koes BW, Boeke AJ, Deville W, De Jong BA, Bouter LM. Shoulder disorders in general practice: prognostic indicators of outcome. Br J Gen Pract J R Coll Gen Pract. 1996;46:519-523.
9. Croft P, Pope D, Silman J. The clinical course of shoulder pain: prospective cohort study in primary care. Br Med J. 1996;313:601-602.

10. Winters JC, Sobel JS, Groenier KH, Arendzen JH, Meyboom de Jong B. The long-term course of shoulder complaints: a prospective study in general practice. Rheumatology 1999;38:160-163.

11. Lui PP, Maffulli N, Rolf C, Smith RK. What are the validated animal models for tendinopathy? Scand J Med Sci Sports. 2011;21:3-17.

12. Arensburg L, Wilderjans H, Simon O, Dewulf J, Boussauw B. Nonseptic tenosynovitis of the digital flexor tendon sheath caused by longitudinal tears in the digital flexor tendons: a retrospective study of 135 tenoscopic procedures. Equine Vet J. 2011;43:660-668.

13. Smith MR, Wright IM. Noninfected tenosynovitis of the digital flexor tendon sheath: a retrospective analysis of 76 cases. Equine Vet J. 2006;38:134-141.

14. Smith MR, Wright IM, Minshall GJ, Dudhia J, Verheyen K, Heinegård D, Smith R. Increased cartilage oligomeric matrix protein concentrations in equine digital flexor tendon sheath synovial fluid predicts intrathecal tendon damage. Vet Surg. 2011;40(1):54-58.

15. Gotoh M, Hamada K, Yamakawa H, Nakamura M, Yamazaki H, Ueyama Y, Tamaoki N, Inoue A, Fukuda H. Perforation of rotator cuff increases interleukin 1beta production in the synovium of glenohumeral joint in rotator cuff diseases. J Rheumatol. 2000;27:2886-2892.

16. Voloshin I, Gelinas J, Maloney MD, O'Keefe RJ, Bigliani LU, Blaine TA. Proinflammatory cytokines and metalloproteases are expressed in the subacromial bursa in patients with rotator cuff disease. Arthroscopy. 2005;21:1076.

17. Demirag B, Sarisozen B, Ozer O, Kaplan T, Ozturk C. Enhancement of tendon-bone healing of anterior cruciate ligament grafts by blockage of matrix metalloproteinases. J Bone Joint Surg Am. 2005;87:2401-2410.

18. Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini FC, Krause DS, Deans RJ, Keating A, Prockop DJ, Horwitz EM. Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy. 2006;8:315-317.

19. Godwin EE, Young NJ, Dudhia J, Beamish IC, Smith RK. Implantation of bone marrow-derived mesenchymal stem cells demonstrates improved outcome in horses with overstrain injury of the superficial digital flexor tendon. Equine Vet J. 2012;44(1):25-32.

20. Smith RK, Werling NJ, Dakin SG, Alam R, Goodship AE, Dudhia J. Beneficial effects of autologous bone marrow-derived mesenchymal stem cells in naturally occurring tendinopathy. PLoS One. 2013;8(9):e75697.

21. Nuver-Zwart I, Schalkwijk J, Joosten LA, van den Berg WB, van de Putte LB. Effects of synovial fluid and synovial fluid cells on chondrocyte metabolism in short term tissue culture. J Rheumatol. 1998;15(2):210-216.

22. Andrish J, Holmes R. Effects of synovial fluid on fibroblasts in tissue culture. Clin Orthop Relat Res. 1979;138:279-283.

23. Brand JA, McAlindon TE, Zeng L. A 3D system for culturing human articular chondrocytes in synovial fluid. J Vis Exp. 2012;31(59):e3587.

24. Sun L, Zhou X, Wu B, Tian M. Inhibitory Effect of Synovial Fluid on Tendon-to-Bone Healing: An Experimental Study in Rabbits. Arthroscopy. 2012;28(9):1297-1305.

25. Garvican ER, Dudhia J, Alves AL, Clements LE, Plessis FD, Smith RK. Mesenchymal stem cells modulate the release of matrix proteins from tendon surfaces in vitro: a potential beneficial therapeutic effect. Regen Med. 2014;9(3):295-308.

26. Dudhia J, Scott CM, Draper ER, Heinegard D, Pitsillides AA, Smith RK. Aging enhances a mechanically-induced reduction in tendon strength by an active process involving matrix metalloproteinase activity. *Aging Cell.* 2007;6(4):547-556.

27. Abràmoff MD. Image processing with ImageJ. *Biophotonics Int*. 2004;11(7):36-42.

28. Dakin SG, Smith RK, Heinegård D, Önnerfjord P, Khabut A, Dudhia J. Proteomic analysis of tendon extracellular matrix reveals disease stage-specific fragmentation and differential cleavage of COMP. *J Biol Chem*. 2014;289(8):4919-4927.

29. Bindra RR. Basic Pathology of the Hand, Wrist and Forearm: Tendon and Ligament. In: Berger RA, Weiss AC, eds. *Hand Surgery*. 2004; Philadelphia: Lippincott, Williams, Wilkins; p 26.

30. Birch HL, Thorpe CT, Rumian AP. Specialisation of extracellular matrix for function in tendons and ligaments. *Muscles Ligaments Tendons J*. 2013;3(1):12-22.

31. Zhou JX, Li X. Apoptosis in Polycystic Kidney Disease: From Pathogenesis to Treatment. In: Li X, ed. *Polycystic Kidney Disease.* 2015; Brisbane (AU): Codon Publications.

Figure Legends

Figure 1. Viability of cells in equine tendon and epitenon explants. Live/Dead staining using Calcein AM/ propidium iodide of equine explants cultured in control culture media (D10) or synovial fluid culture media (SF media); a, b) intact outer epitenon from the compressive region of the DDFT; c,d) core SDFT explant; e,f) core DDFT from the tensile region; g,h) core DDFT from the compressive region. The compressed region of the DDFT is less cellular than the tensile region and cells appear more rounded and do not follow the linear parallel pattern observed by tenocytes in either SDFT or T-DDFT.

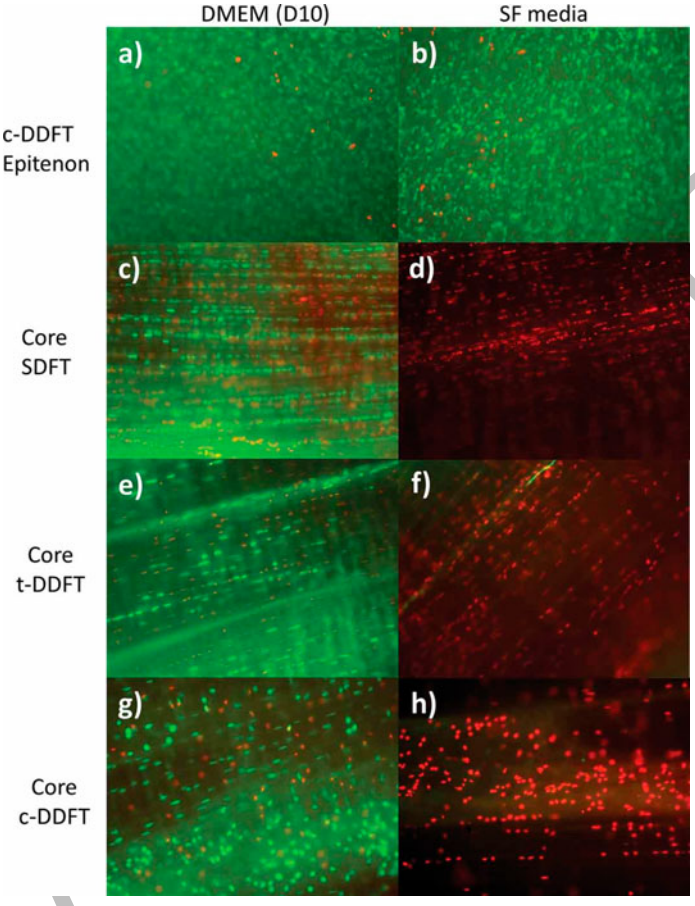

Figure 2. Quantification of equine tendon explant cell viability. Percentage viability of tendon explants from a) SDFT; b) tensile region of the deep digital flexor tendon (T-DDFT); c) compressed region of the deep digital flexor tendon (C-DDFT) cultured for up to 24 h in control culture media (DMEM) or synovial fluid culture media (SF), assayed using Calcein AM/ propidium iodide fluorescent cell staining. Data is an average count from a minimum of three fields of view.

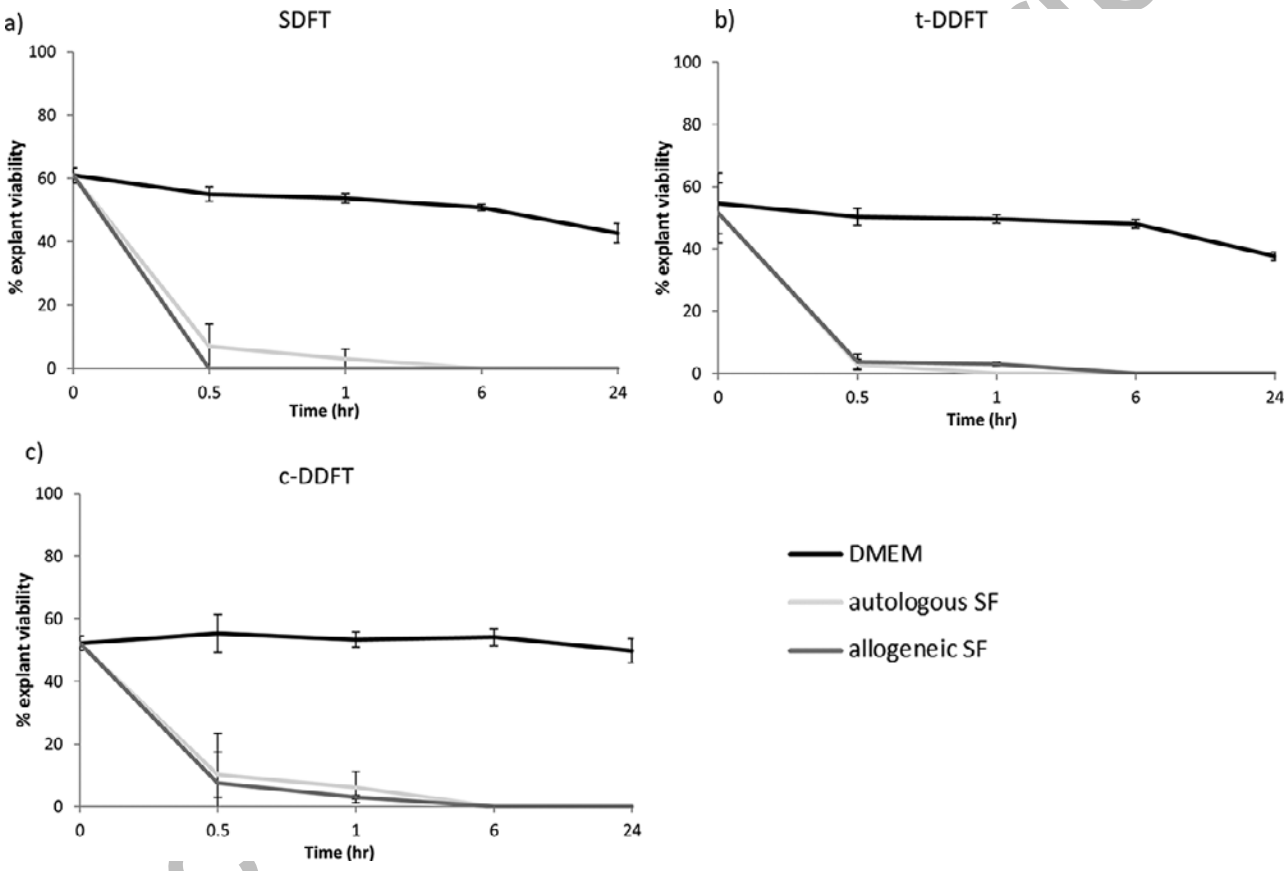

Figure 3. Viability of cells in human hamstring tendon. Live/Dead staining using Calcein AM/ propidium iodide; a, d) hamstring cultured in control media (D20); b, e) hamstring cultured in allogeneic synovial fluid (SF) for 1 h; c, f) blood vessels located within hamstring cultured in allogeneic synovial fluid.

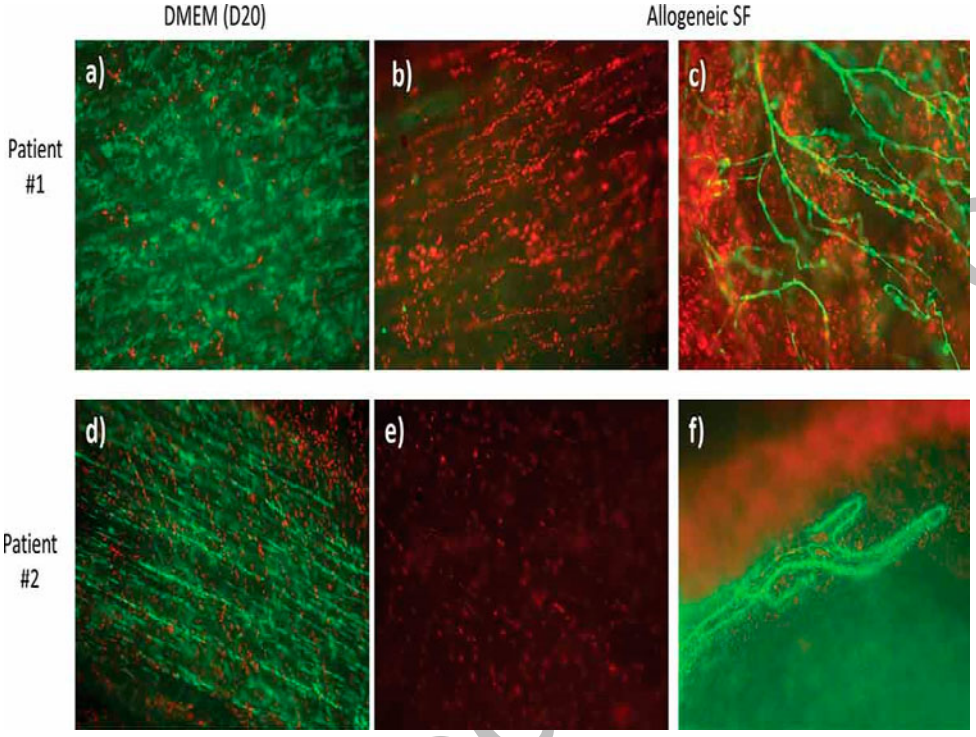

Figure 4. Viability of monolayer equine cells in synovial fluid. a,b) Metabolic activity of MSCs (n = 2) and TDCs (n = 2) cultured in monolayer for 6 days in either control culture media (D10) or synovial fluid culture media (SF), measured using the alamarBlue® assay. c,d) Metabolic activity of MSCs (n = 3) and TDCs (n = 3) co-cultured for up to 24 h with explants derived from the compressed region of the deep digital flexor tendon. In these cultures the alamarBlue® data is referred to as metabolic activity because no significant cell proliferation occurs within the 24 h culture period. However any toxic effect on cells would impact mitochondrial activity (which this assay measures).

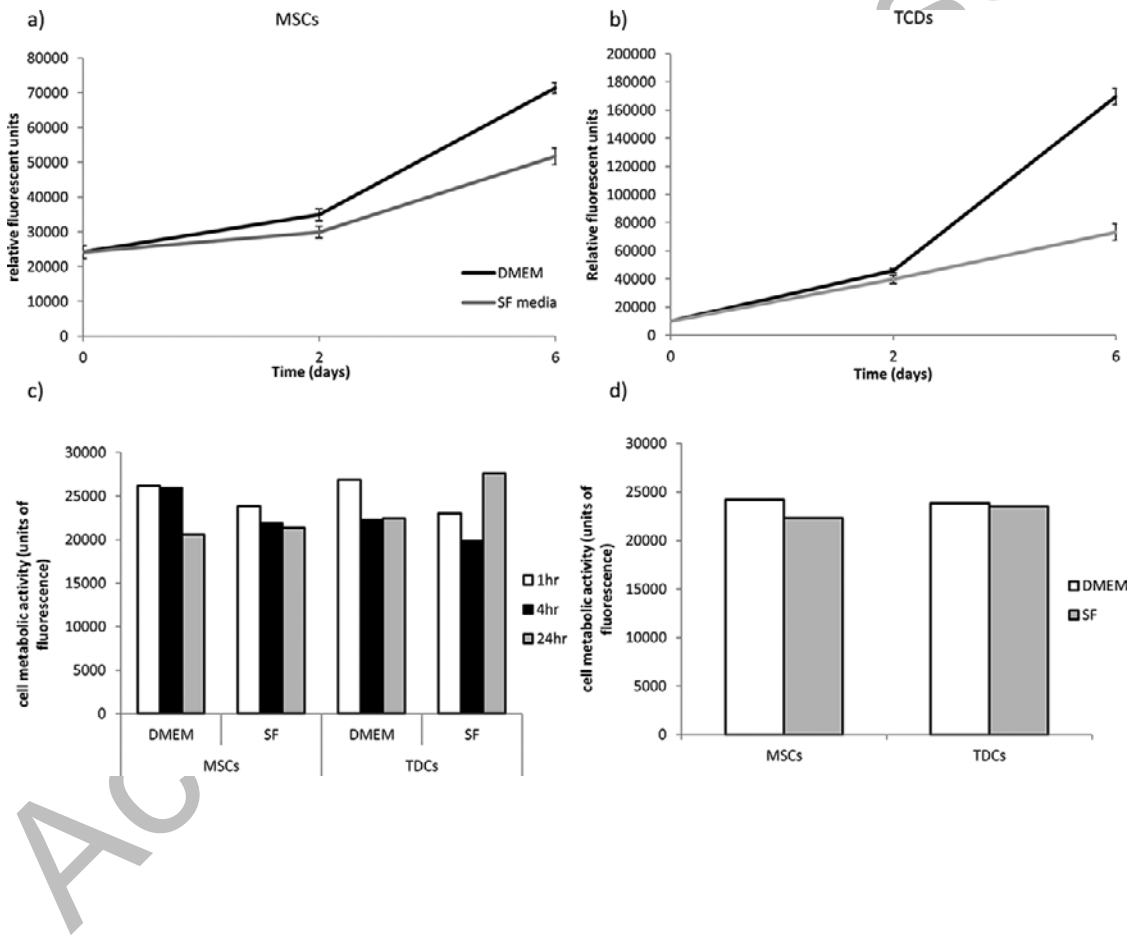

Figure 5. Viability of re-cellularised NNTs. Live/Dead staining using Calcein AM/ propidium iodide of a,b) control (uncoated) C-DDFT explant; c,d) MSCs and; e,f) TDCs cultured for 21 days adhered to C-DDFT explants, then cultured for 1 h in control culture media (DMEM) or synovial fluid culture media (SF).

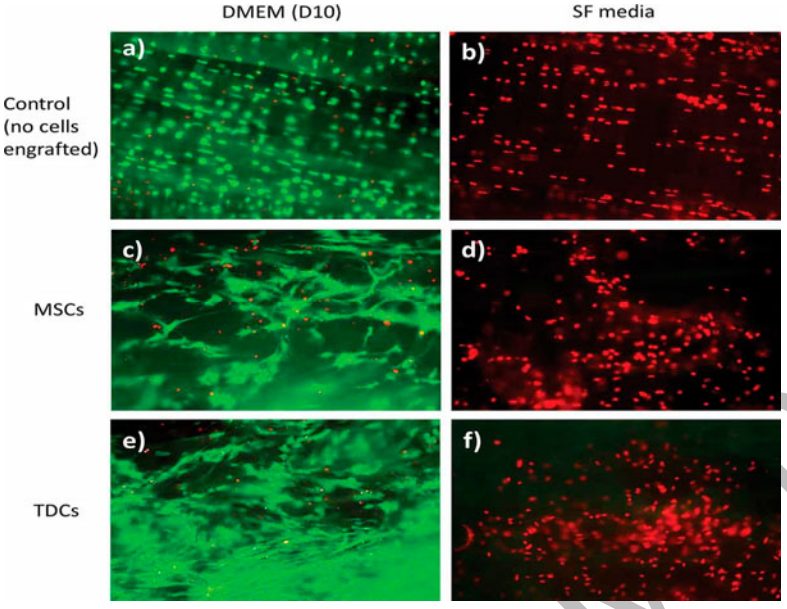

Figure 6. Expression of caspases -3, -8 and -9 by SDFT, T-DDFT and C-DDFT explants cultured for 1 h in control culture media (D10), autologous or allogeneic synovial fluid culture media, expressed as a percentage relative to live cell numbers determined by viability staining from paired samples analysed in Figure 2.