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An in vitro investigation into the effects of 10Hz cyclic loading on tenocyte metabolism

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Abstract

Tendinopathy is a prevalent, highly debilitating condition, with poorly defined aetiology. A wide range of clinical treatments have been proposed, with systematic reviews largely supporting shock wave therapy or eccentric exercise. Characterising these treatments has demonstrated both generate perturbations within tendon at a frequency of approximately 8-12Hz. Consequently, it is hypothesised that loading in this frequency range initiates increased anabolic tenocyte behaviour, promoting tendon repair. The primary aim of this study is to investigate the effects of 10Hz perturbations on tenocyte metabolism, comparing gene expression in response to a 10Hz and 1Hz loading profile.

Tenocytes from healthy and tendinopathic human tendons were seeded into 3D collagen gels and subjected to 15 mins cyclic strain at 10Hz or 1Hz. Tenocytes from healthy tendon showed increased expression of all analysed genes in response to loading, with significantly increased expression of inflammatory and degradative genes with 10Hz, relative to 1Hz loading. By contrast, whilst the response of tenocytes from tendinopathy tendon also increased with 10Hz loading, the overall response profile was more varied and less intense, possibly indicative of an altered healing response.

Through inhibition of the pathway, IL1 was shown to be involved in the degradative and catabolic response of cells to high frequency loading, abrogating the loading response.

This study has demonstrated for the first time that loading at a frequency of 10Hz may enhance the metabolic response of tenocytes by initiating an immediate degradatory and inflammatory cell response through the IL1 pathway, perhaps as an initial stage of tendon healing.

Key words: Tendinopathy, high frequency, IL1 pathway, inflammatory response, 3D collagen gel, cyclic strain.

Introduction

Tendons are fibrous connective tissues, composed of cells within a collagen-rich extracellular matrix (ECM)[1]. Connecting muscle to bone, tendons are passive structures with a force transmission function[2]. The Achilles tendon is the strongest, thickest and largest tendon in the human body, connecting the triceps surae muscle group to the calcaneus[3], and acting as an energy store, stretching to store then release energy during locomotion. Being exposed to higher stresses and strains than other tendons[4], the Achilles tendon is particularly prone to injuries or tendinopathies.

Tendinopathies are associated with significant pain and disability, and remain difficult to treat, owing in part to limited understanding of the underlying aetiology and pathogenesis of the conditions. Treatment options have subsequently varied widely[5]. However, systematic review articles have generally supported the use of extracorporeal shock wave therapy (ESWT) or eccentric loading as two of the more effective treatment options[6,7,8,9,10,11,12]. Recent studies have focused on establishing mechanisms of action for both ESWT and eccentric loading[13,14]. ESWT directly applies mechanical stimulation to tendon in the range of 8-10Hz, whilst biomechanical studies of eccentric exercises have demonstrated that vibrations occur in the lower limb in a similar 8-12 Hz range whilst completing the exercises. With both treatments producing perturbations within the muscle-tendon complex at a frequency of approximately 8 to 12 Hz[13,14,15]. It has subsequently been hypothesised that 8 to 12 Hz perturbation may stimulate tendon repair at a cellular level.

Mechanical loading is one of the regulatory factors known to influence tendon metabolism, with tenocytes responsive to mechanical stimuli through mechanotransduction processes that regulate the expression of both anabolic and catabolic genes, and can lead to maintenance, remodelling or degeneration of the tendon structure[16,17]. Mechanotransduction studies generally adopt isolated cells to investigate cell response to varying strain conditions under more tightly controlled conditions, predominately investigating response to low frequency loading (0.3Hz – 1Hz)[16,18,19]. However, previous studies have demonstrated that housing cells in a 3D environment can provide a more physiologically relevant cell environment as a basis for such investigations[20,21]. For example, using 3D collagen gels, human Achilles tenocytes have been reported to express increases in ADAMTS2/4/16, MMP24, TIMP3 and type I collagen gene expression after 24 or 48hrs loading[22].

Despite a range of mechanotransduction studies using tenocytes, very few studies have investigated the tenocyte response to loading frequencies greater than 1Hz, nor the cell response to the 8-12Hz loading frequency range. Further, the majority of studies investigating tenocyte response to loading have focused on healthy cells, with very little work investigating the response of cells from tendinopathic tissue. Recent studies directly comparing tenocytes from healthy and tendinopathic tendon highlighted differences in the response of the two cell types[23,24], which may be of relevance for understanding repair processes in tendinopathic tendons.

As such, this study is the first to investigate tenocyte metabolic activity in response to 10Hz and 1Hz mechanical loading in cells from both healthy and pathological tendon, to explore if loading frequency modulates cell activity in either cell type. In addition, it aims to further characterise the reported mechanotransduction response, by probing the signalling pathways involved in the mechanoregulation of gene expression. We hypothesise that mechanical loading at 10Hz will lead to an augmented anabolic tenocyte response relative to loading at 1Hz, and that tenocytes from a tendinopathic source will show a different response.

Materials and methods

Construct Preparation

Human tenocytes were derived from tendinopathic Achilles tendon [n = 3, Age: 21 ± 1 years] and healthy semitendinosus tendon [n = 3, Age: 48 ± 3 years] under ethical permission and informed consent (NRES Committee East of England – Essex (REC number 09/H0302/3). Tenocytes were obtained from these tissues by either explant outgrowth for the tendinopathic Achilles tendon or collagenase digest for the healthy semitendinosus tendon at the Bio-Medical Research Centre (BMRC) University of East Anglia (UEA) (Jones, 2012). The cells were grown to ~90% confluence in culture media containing Dulbecco's Modified Eagle's Medium (DMEM) (low glucose, glutaMAX and pyruvate [Life Technologies, Paisley, UK]), foetal bovine serum (FBS) and penicillin/streptomycin (Life Technologies, Paisley, UK) at a ratio of 100:10:1.

Tenocytes were grown to passage 3, where sufficient cells for studies were available, and then seeded into 3D collagen gels, prepared as described previously by Phillips and Brown (2011). To briefly recap preparation of gels, 10X Minimum Essential Medium with phenol red (MEM – Life Technologies, Paisley, UK) was mixed with rat tail collagen (First Link - 2mg/ml chloroform treated, Birmingham, UK) at a ratio of 1:8 and the pH of the collagen solution adjusted to approximately 7 by the addition of 5M sodium hydroxide. The tenocyte suspension and neutralised collagen solution were mixed 1:9 (0.4 mg/ml collagen, 0.1×10^6 cells/ml final density) and 5ml was pipetted into a rectangular mould and allowed to incubate at room temperature for 30 minutes to solidify. The resulting highly hydrated gels were placed between pieces of absorbent filter paper, and a 120g weight placed on top for 5mins to exude water and leave the gels as a 100-200μm thick collagen sheet in a process coined 'plastic compression'[25].

The collagen sheets were rolled into cylinders and secured into the custom designed stainless steel chambers at a grip-to-grip distance of 10 mm[26]. Chambers were filled with DMEM, sealed with a glass surround and maintained in an incubator for 24hrs to allow tenocytes to

stabilise within the new environment prior to the start of any loading experiments. Preliminary experiments confirmed cell viability when constructs were secured in chambers in this manner with and without loading for up to 72 hours.

Mechanical Loading of Tenocytes

A preliminary loading experiment was performed at 10Hz using a Taqman Low Density Array (TLDA) to investigate appropriate time points and genes for further investigation. Ten samples were prepared with tenocytes from healthy tendon, and secured within a custom designed loading frame fitted within a materials testing machine (BOSE Corporation, Eden, Prairie, Minnesota, USA), and housed in an incubator for the duration of the loading experiment (for further detail of the loading equipment see Legerlotz *et al.,* 2013). Two samples were removed after the 24 hours stabilisation period to provide baseline gene expression data. The remaining eight were subjected to 15 minutes of 1% ± 1% sinusoidal cyclic loading at 10Hz, then held statically at 1% strain until the 4, 8, 12 or 24 hour time point (n = 2 per time point) after which the TLDA analysis was carried out to investigate the time course of gene expression across a wide range of matrix genes (Figure 1).

Genes for further analysis were selected from this initial data set, and a consistent analysis time point of 24 hours selected for a series of further loading studies. As such, all studies utilised 1% ± 1% sinusoidal cyclic loading, with samples held under 1% static strain after the cyclic loading period, until the 24 hour analysis time point.

Firstly, the differential effects of 1Hz and 10Hz loading were explored. Collagen gels were seeded with tenocytes from either healthy or tendinopathic tendon (n = 12 per cell type) and split into 3 test groups, each with four technical repeats: unstrained controls, low frequency loading (15mins of 1Hz cyclic loading) and high frequency loading (15mins of 10Hz cyclic loading). The experiment was carried out three times with different biological donors, with resulting gene expression investigated utilising RT-PCR.

By necessity, both loading frequency and the total number of loading cycles differed between groups in this initial experiment, so to control for total number of loading cycles, a second experiment was performed, maintaining a consistent application of 9000 loading cycles to each test group. Collagen gels were seeded with tenocytes from healthy tendon (n = 11) to provide 3 test groups: unstrained controls (n = 3), low frequency loading long (150mins of 1Hz cyclic loading; n = 4) and high frequency loading short (15mins of 10Hz cyclic loading; n = 4). The experiment was repeated three times with different biological donors, and gene expression analysed using RT-PCR.

Finally, the role of inflammatory mediators in the cell response was further examined. First, a preliminary screen of 80 cytokines was carried out to select targets for further analysis. Six collagen gels were prepared with tenocytes from healthy tendon (n=2 per condition): unstrained controls, low frequency (15mins; 1Hz), and high frequency (15mins; 10Hz). A cytokine array (ab133998 - Abcam, Cambridge, UK) was used to analyse protein response at 24 hours following previously described protocols[27]. For each sample, the developed Western blots were subjected to digital densitometry to determine the differences in cytokine expression in each sample relative to the unstrained control. Signal intensity for each cytokine under each loading condition was plotted relative to the control in log base 10, so as

to respond to skewness towards large values (where a few points are much larger than the bulk of the data), making it easier to identify the changes between groups (Supplementary Figure 1 & Table 1). However, a few of the cytokine patterns, where the control sample had no signal, were set to 1, in order to directly compare each cytokine.

Subsequently, the role of IL1 alpha in the tenocyte strain response was investigated. Tenocytes from healthy tendon were seeded into collagen gels and secured within chambers filled with loading media, with or without IL-1 inhibitory antibody, at a concentration of 1 mg/ml media (Abcam, ab9614). The concentrations of IL-1 inhibitory antibody were selected based on previous studies with tenocytes[28]. Loading conditions from the preliminary cytokine screen were repeated, comparing unstrained controls, low frequency (15mins; 1Hz), and high frequency (15mins; 10Hz) loading, both with and without the inhibitor (n=3 for each test condition). The experiment was repeated with three biological repeats of each cell type and gene expression analysed with RT-PCR.

RNA extraction and reverse transcription

At the end of each experiment, gels were removed from chambers by cutting off the gripped ends, and dissolved in 1ml of Trizol reagent (Life Technologies, Paisley, UK). RNA was isolated as described previously using a tri-spin protocol[44] and re-suspended in 30µl of analytical grade water. The RNA concentration of samples was estimated using a NanoDrop spectrophotometer and the absorbance ratio determined as an indicator of RNA quality. RNA was diluted across all samples to match the smallest sample concentration of the experimental set. RNA was primed using random hexamers and reverse transcribed using the superscript II kit (Life Technologies, Paisley, UK) according to manufacturer's instruction.

Quantitative real time PCR

The preliminary Taqman low density array (TLDA) screening of 48 genes covered 7 MMPs, 8 ADAMTS, all 4 TIMP, 4 interleukins, 12 key proteoglycans and 10 collagens, as well as 3 endogenous control genes 18s, GAPDH and TOP1 (Life Technologies, Paisley, UK; Figure 1).

15µl of cDNA was loaded into the fill reservoirs and the plate run according to manufacturer's instructions, using the Applied Biosystems 7900HT Real-Time System and Applied Biosystems Sequence Detection Systems (SDS) software (Life Technologies, Paisley, UK).

For further experiments, qRT-PCR was run using the selected primer probe sets (see Table 1) on an Applied Biosystems 7500 Taqman system. Each reaction was performed in a volume of 25µl including; cDNA of 1ng/µl, 50% KAPA Probe fast qPCR kit Mastermix (2×) (Anachem, Bedfordshire, UK), 10nM each of the forward and reverse primer and 5nM of probe. Standard curves were run for each assay to confirm primer probe efficiency. Relative expression levels of each gene of interest were analysed by normalising to the endogenous control gene 18s (GeNorme analysis demonstrated 18s to be the most stable housekeeping

gene) and data expressed as $2^{\Delta\Delta Ct}$. All data were normalised to the control, which is represented by the baseline value of 1.

Statistical analysis

Sample data were checked to confirm normality, after which statistical analysis was performed using a one-way analysis of variance (one-way ANOVA), looking for significant differences between the responses of tendinopathic and healthy tendon cell types (experiment 1), the response to different loading conditions (experiment 2) and the effects of the inhibitor (experiment 3). Tukey's Honest Significant Difference (HSD) post-hoc tests were adopted where significant differences were identified. P values < 0.05 were considered significant.

Results

The preliminary TLDA data set indicated peak gene expression at 24 hours, ensuring this time point was selected as the analysis time for all future experiments (figure 1). Of the analysed genes, MMP7, MMP8, ADAMTS3, COL2A1 and COL9A1 were not detected. The large majority of all remaining genes showed a steady increase in gene expression to a peak expression at 24hrs. The metalloproteinase and matrix genes selected for further analysis were chosen based on their regulation with strain, in addition to knowledge of their key role in tendon matrix turnover. Fold changes for the selected genes in response to 10Hz cyclic strain at 1% ± 1% were: MMP1 (7.3), MMP13 (5.1) ADAMTS5 (10.0), IL6 (8.1), IL8 (5.4), COL1A1 (6.0), COL3A1 (5.4), and COL5A1 (566.3). All fold changes are shown in Figure 1.

A comparison of tenocyte response to 1Hz and 10Hz loading demonstrated that gene expression changes were stimulated by both 10 Hz and I Hz loading, although there was a greater response to 10 Hz loading (compared to 1 Hz) in some genes for cells from both healthy and tendinopathic tendon (Figure 2A and B). Large error bars highlight the notable variability between biological replicates, but housekeeping gene levels demonstrated consistency between cell types (supplementary table 2). Paired analyses of data indicated significant differences in response were evident in genes from the healthy tendon cell population only, specifically MMP1, IL6 and IL8, which were all significantly more responsive to 10Hz than 1Hz loading (Figure 2B). It was notable that the overall gene expression profile differed between cell types, and whilst healthy tendon cells showed upregulation of all genes investigated with load, tendinopathic tendon cells showed a downregulation of COL1A1 and COL5A1 with loading. It was also notable that substantially higher gene expression levels were consistently seen in the tenocytes from healthy compared to tendinopathic tendons.

Selected genes were analysed to look more specifically at the basis for the differential cell response to 10Hz and 1Hz loading. An additional loading group, applying 1Hz loading for 150 minutes was included, matching the number of loading cycles applied to that in the 10Hz loading group, and enabling the effects of loading frequency, loading time and loading cycles to be more simply distinguished. Once again, data highlighted no significant differences in collagen gene expression between any of the loading groups (Figure 3). By contrast, the

inflammatory and degradatory genes under investigation (MMP1, MMP13, IL-6, IL-8 and ADAMTS5) showed clear differential responses when comparing 9000 cycles of loading at 10Hz and 1Hz with significantly upregulated gene expression for MMP1, IL-6 and IL-8 with 10Hz loading (Figure 3).

Finally the cell response to 1Hz and 10Hz loading was investigated with and without an IL-1 inhibitor to ascertain the possible role of Il-1 in the cell strain response. The addition of the IL-1 inhibitor significantly reduced the load induced expression of MMP13, but notably it completed abrogated the expression of MMP1 and IL-6, to levels below those seen in the unloaded control samples (Figure 4).

Discussion

This study is the first to investigate tenocyte response to 10Hz loading within a 3D environment. The focus on investigating cell metabolism at this frequency stems from a goal to explore how the vibrations likely seen in the tendon during therapeutic modalities of proven efficacy, such as ESWT and eccentric loading, may impact cell mechanobiology. We found that 1% ± 1% strain applied at 10Hz initiated a greater catabolic response in cells from both healthy and tendinopathic tendons, relative to that seen with loading at 1Hz, with a significant upregulation of inflammatory (IL6 and IL8) and degradative genes (MMP1) seen in cells from a healthy source. By contrast, changes in collagen expression were not evident. Within the timeframes investigated, this disputes the hypothesis that 10Hz loading initiates increased anabolic tenocyte behaviour. However, it is well known that both catabolic and anabolic metabolism are necessary for matrix turnover and renewal, so it may well still be that the response investigated at this snapshot in time is part of an accelerated tissue remodelling response to mechanical stimulation. Of further note, it was also evident that the load response was more robust in cells from healthy than tendinopathic tendon. Finally, this study suggests that tenocytes respond to loading at least in part through the IL-1 signalling pathway. Further exploration of this pathway may identify mechanisms to simulate or augment the adaptation response seen with mechanotherapy-based treatments.

There have been a number of studies that have looked at the effects of cyclic strain on tenocytes, applied at varying low frequencies. It is difficult to compare studies of different design to ascertain how loading frequency may influence cell response. However, the few studies which have directly investigated cell response to loading at more than one frequency suggest a more pronounced response with higher frequency loading, consistent with this current study[18,19,29,30]. These studies demonstrated increased expression of type I and type III collagen, as observed in the current study, and also reported increased expressions of tenascin-C, tenomodulin and scleraxis. However, 1Hz loading generally denoted the fastest loading speed in these studies, and cell metabolism in response to higher frequencies has not previously been reported.

Our focus on 10Hz loading reflected an interest in interrogating the potential role of the 8-12 Hz frequency range in stimulating cellular metabolism and tendon repair during ESWT or eccentric loading exercises. Indeed, it would be interesting to determine if similar 8-12 Hz vibrations are seen in tendon during other proposed loading regimes, such as heavy, slow loading[31]. Comparison of in vivo and in vitro conditions is undoubtedly challenging and care must be taken in attempts to sensibly interface these approaches. Whilst the in vitro approach

offers a simplified environment in which to explore more specific aspects of the complex in vivo condition, the cells are removed from their native environment and a collagen gel is likely to offer a notably less stiff matrix for cell attachment with altered local architecture.

The 10Hz loading frequency was directly adopted for in vitro tests, to mimic the vibrations seen in vivo. However, whilst the frequency of vibrations during these activities has been inferred from previous studies, the magnitude of vibrations remains unknown. A strain magnitude of 1% ± 1% was subsequently adopted, selected as vibrations are by nature of small magnitude, and strains of this range have previously been identified to be highly physiological in tendon at a cell level, occurring when global tendon strains in the range of 5-8% are applied[32]. Previous studies have indicated that altering either the magnitude or duration of loading is likely to influence the resulting cellular metabolism as well as loading frequency[33]. However, these strain conditions, applied for a duration of 15mins were thought to best offer a reasonable imitation of the tendon strain environment during a single bout of eccentric loading, 3 sets of 15 repetitions[34,35].

Owing to the challenges in acquiring human tissue, the current study compared the metabolic response of tenocytes from healthy semitendinosus tendon and tendinopathic Achilles tendon, as available sources. Whilst this comparison is of interest as a mechanism to probe differences in cells from healthy and tendinopathic tissue, care must be taken. Differences in tendon source and mean donor age between groups may also influence cell behaviour, in addition to differences in cell extraction technique (collagenase versus explant growth). However, both the semitendinosus and Achilles tendons exhibit energy-storing capabilities, making them a reasonable functional match despite the age distinction[36].

The cells from healthy semitendinosus tendon demonstrated an upregulation of all analysed genes with loading at either 10Hz or 1Hz frequency, which may indicate a general anabolic response to mechanical loading[37]. By contrast, the metabolic profile of tenocytes from tendinopathic Achilles tendon was more variable, with a subset of genes responding and greater variability between biological repeats. The cell response was also less robust, with less pronounced gene expression levels, and less differentiation in the response to 1Hz and 10Hz loading. It is possible that the greater variability between biological replicates in cells from tendinopathic tendon reflects the likely variability in disease condition between donors. Further, the preferential upregulation of COL3A1 relative to COL1A1 and COL5A1 may denote an early healing response[37], with type III collagen identified as a major component of newly synthesized ECM in early tendon healing[38].

A less active metabolic response from the tenocytes from tendinopathic tendon was also observed by Jones *et al.* (2013), when these same cell populations were subject to 5% cyclic strain at 1Hz in collagen gels using a flexcell system. Both of these studies adopted 3D models, and it is unclear how this may relate to the 2D response. However, the same cells have been shown to behave in a different manner when subject to an alternative loading protocol by Patel et al. Here the cells were seeded into a specially designed fibre composite construct, to applied multi-modal tensile and shear load to the cells at 1Hz for 24hrs. Under these loading conditions, the cells from tendinopathic tendon were more mechano-sensitive[23,24]. Variability in the mechanobiology response of the cells across these studies highlights the importance of physical cues in guiding cell response, suggesting that either the material, how the cell interacted with the material, or the use of shear loading could contribute to the differences seen between the studies.

The more marked metabolic response to 10Hz loading was most evident in the cells from healthy tendon. Other studies have also demonstrated that higher frequency loading (4Hz compared to 1Hz, and 0.2Hz compared to 0.02Hz) better maintained tendon tissue biomechanical properties[29] and accelerated tendon healing response[39] respectively. However, the current data additionally showed that frequency mediated differences in the cell metabolic response were only significant for specific genes (MMP1, IL6 and IL8), with very little frequency-mediated difference in the expression of collagen genes. The significantly greater response of collagenases and cytokines to 10Hz loading may suggest that higher loading frequencies are better able to initiate matrix breakdown. Early matrix breakdown and the initiation of an inflammatory cell response are hypothesised to be crucial for later matrix turnover and repair, suggesting that this initial inflammatory response may be of importance to long term tendon health. Indeed, a previous ESWT study has highlighted a rapid inflammatory and catabolic response in the tendon to ESWT at the protein level[13], suggesting that mechanical stimuli in the frequency range of 8-12Hz might aid tendon remodelling by promoting degradative processes that are associated with removing damaged matrix constituents. A review of protein level response in the current system would be of benefit to more fully interpret cell mechanobiology.

The number of loading cycles applied to cells has also previously been reported to be of importance in modulating cell metabolic behaviour. A recent study showed that increasing the number of loading cycles increased collagen production in the avian flexor digotorum profundus tendon, loaded to either 12MPa or 3MPa at 1Hz for 0, 7200, 43200, 79200 or 86400 cycles[40]. In addition, when rat patellar tendon was cyclically loaded *in vivo* to 50% maximal load, data demonstrated greater changes in gene expression for MMPs, TIMPs, and collagens after 7200 cycles of loading than after 100 cycles[37]. To differentiate between the effects of loading frequency and total number of loading cycles in the current study, an additional experiment was carried out, comparing cells loading at 1Hz and 10Hz for the same total cycle stimulus of 9000 cycles. Inflammatory and degradative markers were again significantly upregulated with 10Hz loading relative to 1Hz loading, suggesting that the metabolic differences reported with loading frequency are robust, and persist irrespective of changes in loading period or total cycle number. However, the notable variation in cell response to loading frequency, loading period and loading conditions all remind us that cell mechanotransduction is complex.

To further probe cell metabolic response, the mechanotransduction pathways associated with cell behaviour were probed. A preliminary screen of cytokine response to 15mins loading at 10Hz and 1Hz (Supplementary figure 1 & Table 1) highlighted that in general, 10Hz loading resulted in greater expression of interleukins, growth factors and angiogenin compared to 1Hz loading. Differences were particularly evident in cytokines previously demonstrated to be associated with IL1 pathways, with IL1 additionally previously indicated to have a role in tenocyte mechanotransduction. IL1 has been reported to show a synergistic effect with mechanical loading[41,42], contributing to the positive metabolic activity necessary for tissue repair, by stimulating increased expression of matrix metalloproteinases (MMPs) such as MMP1, MMP3 and MMP13 in tenocytes[43,44]. Hence, the IL1 signalling pathways was investigated in the current study, to ascertain it involvement in the tenocyte response to high frequency loading.

Inhibiting IL-1 abrogated the expression of collagenase (MMP1 and MMP13) and interleukin (IL6) in response to loading, but had no effect on collagen gene expression. Although, collagenase activity itself was not evaluated, this preliminary study suggests that IL-1 is

involved in the load-mediated degradation of collagen, and it is possible that the modulation in IL-1 production seen with different loading frequencies directly influences the magnitude of catabolic cell behaviour. Anabolic and catabolic pathways are implicated in tendon repair, however, the interactions between the two are complex. IL-1 inhibition has previously been reported to regulate MMPs and IL6[46,47]. These responses are analogous to those seen in this model with IL-1 and strain, further supporting the findings that IL-1 may play a key role in the strain regulation of inflammatory and matrix turnover genes.

Perspectives

This study has demonstrated for the first time that cyclic mechanical strain applied to tenocytes from both healthy and tendinopathic tendon regulates gene expression, primarily for degradative and inflammatory genes, with little response from anabolic genes such as collagens. The response appeared more pronounced with 10Hz loading than seen with more traditionally adopted 1Hz loading regimes, and was also demonstrated to be regulated through an IL-1 signalling pathway. 10Hz vibrations are inferred to occur within tendon during more than one of the tendinopathy treatment regimes with good evidence for efficacy. This study provides a first investigation into the possible mechanisms through which these treatments may be effective, suggesting the vibrations may initiate an immediate degradatory and inflammatory response from the tendon cells. It would be of interest to investigate the local cell strain environment during other treatment regimes of interest, such as heavy, slow loading, to see if similar vibrations are evident during these exercises. Such a cellular response may be the key first step in repair, facilitating matrix turnover towards repair.

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Figure Legends

Figure 1: Regulation of gene expression with mechanical load over the 24hrs loading period in tenocytes from healthy human tendon. Collagen gels were fixed to a custom-made chamber and subjected to a cyclic load of 1% ± 1% for 15mins at 10Hz then statically strained at 1% until the 4, 8, 12 or 24hrs time point. Matrix genes expression levels at each time point were measured using TLDA. Data was normalised to 18s and expressed relative to the 0hr unstrained samples. The greatest changes in gene expression were seen at 24hrs [n = 2 for each time point].

Figure 2: Gene expression changes in tenocytes from A) healthy human semitendinosus tendon and B) tendinopathic human Achilles tendon seeded in 3D collagen gels subjected to a cyclic load of 1% ± 1% for 15mins at 10Hz then held at 1% strain until the 24hr time point. Matrix genes were measured using standard qRT-PCR. Data was normalised to 18s and presented as a fold change relative to the 24hr control (unloaded) samples ($2\Delta\Delta Ct$) [mean ± SD / n = 12 per cell type].

Figure 3: Gene expression changes in tenocytes from healthy human semitendinosus tendon seeded in 3D collagen gels subjected to a cyclic load of 1% ± 1% for 9000 cycles, then held at 1% strain until the 24hr time point. The response to 10Hz and 1Hz loading is compared. Matrix genes were measured using standard qRT-PCR. Data was normalised to 18s and presented as a fold change relative to the 24hr control (unloaded) samples ($2\Delta\Delta Ct$) [mean ± SD / n = 11].

Figure 4: Gene expression changes in tenocytes from healthy human semitendinosus tendon seeded in 3D collagen gels subjected to a cyclic load of 1% ± 1% for 15mins then held at 1% strain for 24hrs. The response to 10Hz and 1Hz loading is compared with and without the addition of IL-1alpha inhibitor. Matrix genes were measured using standard qRT-PCR. Data was normalised to 18s and presented as a fold change relative to the 24hr control (unloaded) samples ($2\Delta\Delta Ct$) [mean ± SD / n = 12]

Table 1: Human primer sequences (Forward and reverse) used for real-time polymerase chain reaction

Target Gene	Primer / Probe Type	Sequence
18s	Forward Reverse Probe	GCCGCTAGAGGTGAAATTCTTG CATTCTTGGCAAATGCTTTCG ACCGGCGCAAGACGGACCAG
MMP1	Forward Reverse Probe	TTTGATGTACCCTAGCTACACCTTCA AAAGGTTAGCTTACTGTCACATGCTTT CCAAGCCATATATGGACGTTCCCAAAATCC
MMP13	Forward Reverse Probe	CCGAGGAGAAACMATGATCTTT GTTAAAAACAGCTCYGCWTCAACCT AGATTCTTCTGGCGSCTGCATCCTC
COL1A1	Forward Reverse Probe	CGCACGGCCAAGAGGAA CATGGTACCTGAGGCCGT TCT CCAAGACGAAGACATCCCACCAATCACC
COL3A1	Forward Reverse Probe	AATAAACTTCAACACTCTTTATGATAACAA CA ACTGGTGAGCACAGTCATTGCT TGTGTTATATTCTTTGAATCCTAGCCCATCT GCA
COL5A1	Primer/Probe Mix	Hs00609133_m1 – Life Technologies
ADAMTS5	Forward Reverse Probe	AGGAGCACTACGATGCAGCTATC CCCAGGGTGTCACATGAATG TGCCCACATAAATCCTCCCGAGTAAAC A
IL6	Forward Reverse SYBR Green	GGTACATCCTCGACGGCATCT GTGCCTCTTTGCTGCTTTCAC
IL8	Forward Reverse SYBR Green	AAGAGAGCTCTGTCTGGACC GATATTCTCTTGGCCCTTGG

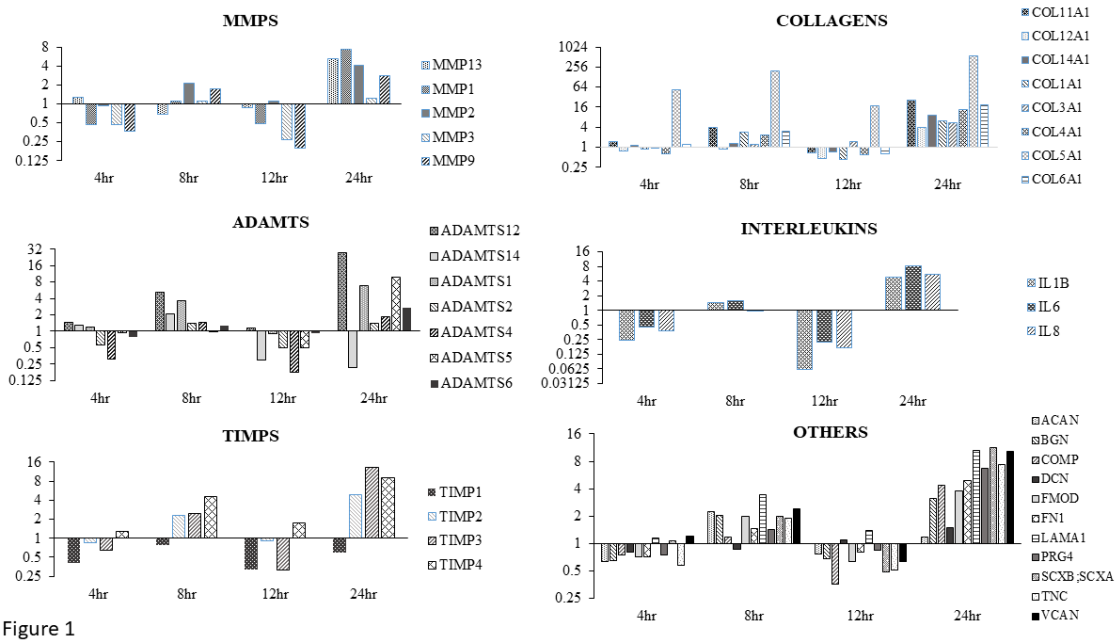

Figure 1

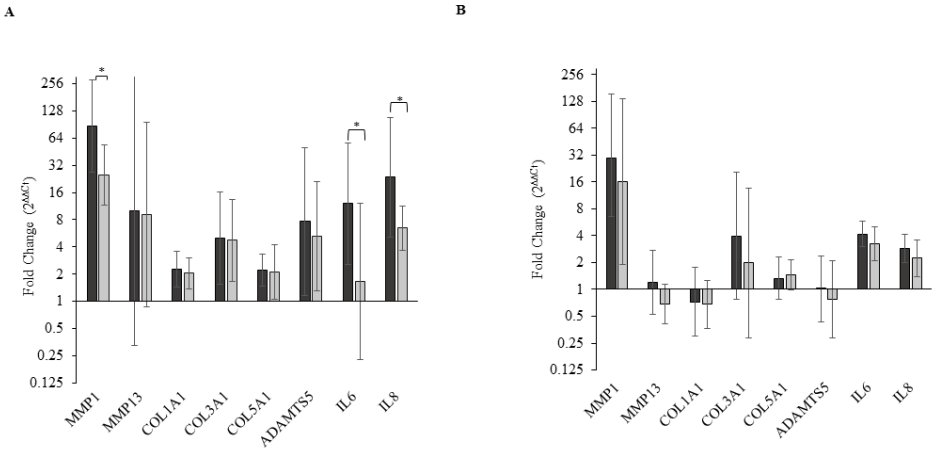

Figure 2

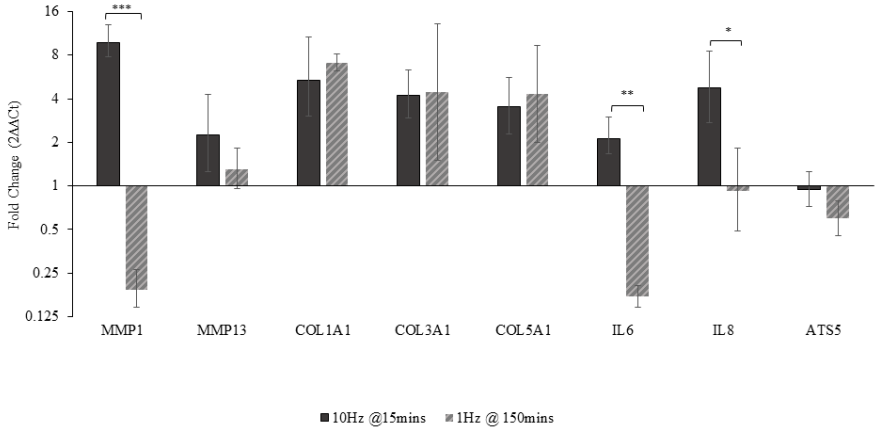

Figure 3

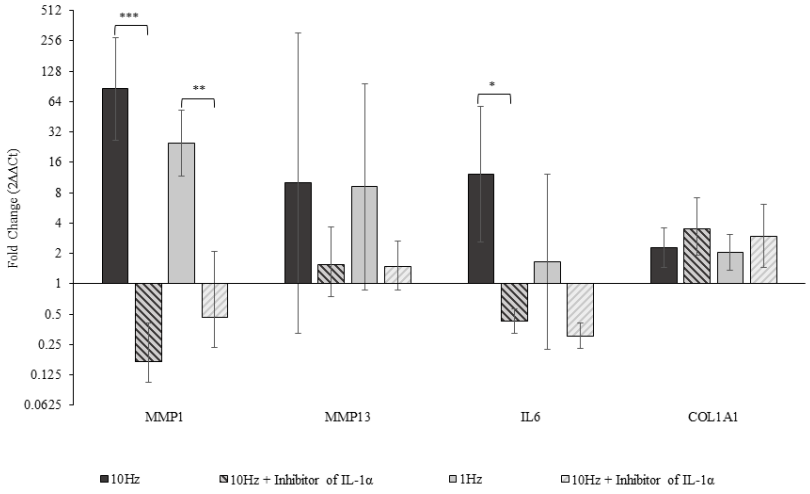

Figure 4