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Tendon overload results in alterations in cell shape and increased markers of inflammation and matrix degradation

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Tendon injury is thought to involve both damage accumulation within the matrix and an accompanying cell response. While several studies have characterized cell and matrix response in chronically injured tendons, few have assessed the initial response of tendon to overload-induced damage. In this study, we assessed cell response to cyclic loading. Fascicle bundles from the equine superficial digital flexor tendon were exposed to cyclic loading *in vitro*, designed to mimic a bout of high-intensity exercise. Changes in cell morphology and protein-level alterations in markers of matrix inflammation and degradation were investigated. Loading resulted in matrix damage, which was accompanied by cells becoming rounder. The inflammatory markers cyclooxygenase-2 and interleukin-6 were increased in loaded samples, as were matrix metalloproteinase-13 and the collagen degradation marker C1,2C. These results indicate upregulation of inflammatory and degradative pathways in response to overload-induced *in vitro*, which may be initiated by alterations in cell strain environment because of localized matrix damage. This provides important information regarding the initiation of tendinopathy, suggesting that inflammation may play an important role in the initial cell response to tendon damage. Full understanding of the early tenocyte response to matrix damage is critical in order to develop effective treatments for tendinopathy.

Tendon injuries are one of the most common musculoskeletal injuries occurring to both athletes and the general population (Clayton & Court-Brown, 2008). Chronic overuse injury is thought to occur because of damage accumulation over the course of a number of loading cycles rather than because of a single overload event (Riley, 2008). It is likely that the accumulation of microdamage leads to alterations in cell behavior; however, this is very poorly understood. The reason etiology is so unclear is because patient samples are usually only available once injury is chronic. In order to fully understand the initiation and progression of tendinopathy, it is important to characterize the molecular events associated with initial damage; therefore, early response to injury has not been fully elucidated.

The role of inflammation in tendon injury is also yet to be fully determined. Several studies have previously failed to identify any inflammatory markers within tendinopathic tissue (Khan et al., 1999; Alfredson & Lorentzon, 2002). However, these samples are often obtained several months post-injury and therefore do not reflect the changes associated with the early stage of tendon injury. More recent work has demonstrated the presence of a number of inflammatory components in acute tendon injury (Dakin et al., 2012b; Manning et al., 2014), leading to the suggestion that tendon cells initiate an early inflammatory cascade to promote healing of the tissue (Dakin et al., 2014). In support of these findings, cyclooxygenase-2 (COX-2) and interleukin-6 (IL-6) expression has been shown to increase in ruptured Achilles tendons (Legerlotz et al., 2012) and levels of IL-6 are also increased in healing tendon, 2 weeks post-rupture (Ackermann et al., 2013). *In vitro* work also shows increased IL-6 gene expression after cyclic loading of tendon fascicles (Legerlotz et al., 2013b), and increased COX-2 expression in a tendon transection animal model has also been reported (Manning et al., 2014).

While the role of inflammation in tendon injury is still debated, it is well established that injury results in alterations in the levels of several of the matrix metalloproteinases (MMPs) within tendon (Jones et al., 2006; de Mos et al., 2007; Riley, 2008), which degrade specific components of the extracellular matrix. Studies have demonstrated alterations in the expression levels of several MMPs occurring immediately post-loading, both

in vitro (Lavagnino et al., 2006; Asundi & Rempel, 2008a) and *in vivo* (Sun et al., 2008; Andarawis-Puri et al., 2012). The majority of these studies have assessed alterations in levels of the collagenases MMP-1 and -13 (Lavagnino et al., 2006; Sun et al., 2008), and the stromelysin MMP-3 (Asundi & Rempel, 2008a). MMP-1 and -13 are likely to play an important role in the early stages of degradation and remodeling of damaged collagen fibers within the matrix. MMP-3 is ubiquitously expressed in tendon tissue, with greater expression levels than MMP-1 and -13 (Birch et al., 2008). This enzyme is able to degrade many minor proteins within the tendon matrix, including collagens III, IV, IX and X, proteoglycans, and elastin, and also is involved in the activation of other MMPs (Woessner, 1991). MMPs-1, -13, and -3 are therefore suitable candidates to include in the study of early response to microdamage.

The horse provides a well-established model to study the initiation and progression of tendon injury as the equine superficial digital flexor tendon (SDFT) shows a high degree of similarity to the human Achilles tendon in terms of function, injury risk and pathological changes (Innes & Clegg, 2010; Lui et al., 2010). Both the equine SDFT and human Achilles act as energy stores, and are therefore exposed to high stresses and strains during exercise (Stephens et al., 1989; Biewener, 1998; Lichtwark & Wilson, 2005). Not surprisingly, both these tendons are prone to damage, with high incidences of clinical injury (Clayton & Court-Brown, 2008; Ely et al., 2009). In the current study, we cyclically loaded explants from the equine SDFT to assess tenocyte response to cyclic loading. We measured changes in cell shape, and used immunofluorescence to assess markers of inflammation and degradation. We hypothesized that cyclic loading would result in cell rounding along with increased levels of the inflammatory markers COX-2 and IL-6, and would also increase levels of the matrix degrading enzymes MMP-1, -3, and -13, resulting in increased collagen degradation.

Methods

Forelimbs distal to the carpus were collected from horses aged 3 to 6 years ($n = 6$) euthanized at a commercial equine abattoir. Prior to dissection, the limbs were stored at 4 °C to minimize proteolytic activity and the SDFT was harvested from each limb within 24 h of death. Tendons were examined macroscopically to ensure there were no signs of injury and experiments performed immediately after tendon dissection to maintain tissue viability.

Assessment of cell morphology in response to loading

To assess the immediate effect of cyclic loading on cell shape, fascicle bundles, approximately $0.5 \times 0.5 \times 25$ mm in dimension, were isolated from the mid-metarcarpal region of the tendon using methods similar to those previously described (Thorpe et al., 2014b). Each bundle contained approximately 8–10 fascicles and six bundles from three SDFTs were used. During the dissection process, tendon hydration was maintained using sterile Dulbecco's Modified Eagle Medium (DMEM). The six fascicle bundles from each tendon were divided into two groups, three control, and three loaded. Controls remained unloaded and were maintained in DMEM for the duration of the experiment. Loaded samples were secured in custom-made loading chambers (Legerlotz et al., 2013b) with a grip to grip distance of 10 mm and were subjected to 1800 loading cycles from 2% to 12% strain (approximately 60% of failure strain) at a frequency of 1 Hz using an electrodynamic test instrument (Electropuls E1000, Instron, Norwood, Massachusetts, USA). Samples were maintained in DMEM at room temperature, under normal environmental conditions for the duration of the test. We have previously demonstrated that application of a similar loading protocol is sufficient to induce fascicle damage, characterized by fiber kinking and widening of the interfiber space (Thorpe et al., 2014a, b). Force and displacement data were recorded at a rate of 100 Hz. Throughout loading, samples were immersed in sterile DMEM. After loading, the clamped ends of the samples were removed and the samples were incubated in calcein AM (20 µL/mL DMEM) for 2 h at 37 °C to stain the cells. Samples were briefly washed in DMEM, then viewed under a confocal microscope (Leica TCS SP2, Leica Microsystems, Wetzlar, Germany). Images were taken in the central region of the fascicle bundles, using × 20 air objective.

Image analysis

To assess changes in cell shape, a grid measuring 10 by 10 squares was superimposed on each image and a random number generator was used to select 10 squares, covering an area of 13 590 µm^2. The length and width of all cells in the selected squares were measured using ImageJ (1.44, NIH, Bethesda, Maryland, USA) and the cell aspect ratio calculated by dividing cell length by width. To avoid observer bias, the assessor carrying out the cell aspect ratio measurements was blinded to the test condition.

Assessment of cell inflammatory response to loading

Loading protocol

To assess the cell response to cyclic loading, fascicle bundles were dissected from the mid-metacarpal region of the SDFT as described above ($n = 3$ tendons, nine samples per tendon). Fascicle bundles were divided into three groups as follows: fresh frozen control, static control, and loaded ($n = 3$ for all groups). Fresh frozen controls were embedded in optimal cutting temperature (OCT) compound and snap frozen in hexane cooled on dry ice immediately post-dissection. Static controls and loaded samples were secured in the loading chambers, and static controls were subjected to 2% applied strain for 24 h, while samples in the loaded group underwent 1800 loading cycles from 2% to 12% strain at a frequency of 1 Hz and were then maintained at 2% applied strain for the remainder of the 24 h period. A Bose mechanical test system housed in an incubator was used to apply the loading regime to the samples (Wintest v 4.0, Bose Corporation, Eden Prairie, Minnesota, USA). Throughout the loading period, all samples were immersed in sterile DMEM supplemented with penicillin (50 U/mL), streptomycin (0.05 mg/mL) and L-glutamine (2 mM) and maintained at 37 °C, 20% O_2, 5% CO_2. After 24 h, samples were removed from the loading chambers and the clamped ends of the fascicle bundles were removed and discarded. Preliminary experiments demonstrated cell death at the cut edges of the fascicle bundle after 24 h of culture, but good cell viability (greater than 80% as determined by calcein AM and ethidium homodimer staining) in the core of the bundle, so sample

Table 1. Details of primary and secondary antibodies used for immunostaining

Protein	Primary antibody	Concentration	Secondary antibody	Concentration	Validation
Cox-2	Mouse monoclonal anti-Cox-2 (Cayman Chemical 160112)	1:50	Donkey anti-mouse 488	1:500	(Menzies-Gow et al., 2008)
IL-6	Goat polyclonal anti-IL6 (R&D systems AF1886)	1:25	Donkey anti-goat 543	1:500	(Ley et al., 2009)
C1,2C	Rabbit polyclonal anti-C1,2C (IBEX 50-1035)	1:20	Donkey anti-rabbit 488	1:500	(Billinghurst et al., 1997) (Frisbie et al., 2008)
MMP-1	Goat polyclonal anti-MMP1 (Santa Cruz 6837)	1:50	Donkey anti-goat 543	1:500	See Supporting Information
MMP-3	Goat polyclonal anti-MMP3 (Abcam 18898)	1:25	Donkey anti-goat 543	1:500	(Peffers, 2013; Peffers et al., 2013)
MMP-13	Rabbit polyclonal anti-MMP3 (Santa Cruz 30073)	1:50	Donkey anti-rabbit 488	1:500	See Supporting Information

edges were also removed by sharp dissection and the core of the samples (2–3 fascicles) embedded in OCT and snap frozen in hexane cooled on dry ice.

Immunostaining

Longitudinal cryosections 20 μm thick were cut from each sample and placed on poly-lysine slides. Slides were stored at – 80 °C until required. Prior to immuno-staining, slides were thawed and fixed with ice-cold acetone for 10 min. Slides were rinsed in phosphate buffered saline, then washed in Tris buffered saline with 0.1% Triton-X (TBS-T). Sections were incubated in blocking buffer (TBS with 1% bovine serum albumin, 10% donkey serum) for 2 h. Samples were drained, and primary antibodies, diluted in blocking buffer, were applied to each section and incubated overnight at 4 °C. The antibodies used and their concentration are detailed in Table 1. Samples were dual stained for (a) COX-2 and IL-6; (b) MMP-3 and -13; and (c) MMP-1 and the collagen degradation marker C1,2C, which recognizes the carboxy terminus of the three-quarter collagen fragment produced by collagenase cleavage (Billinghurst et al., 1997). Antibodies that had not been previously validated in equine tissue were validated using Western blotting (see Supporting Information). Negative controls were included, in which the primary antibody was omitted from the blocking buffer; no nonspecific staining was observed in any of these samples. After incubating overnight, sections were washed twice in TBS-T, and FITC- or TRITC-conjugated secondary antibodies diluted in blocking buffer were applied and incubated for 2 h at room temperature in the dark (see Table 1 for details). Sections were washed and mounted with ProLong Gold Antifade reagent with 4′,6-diamidino-2-phenylindole (DAPI). Samples were allowed to cure overnight at 4 °C before being sealed and imaged.

Imaging was performed on a confocal microscope (Leica TCS SP2) using × 20 (air) and × 40 (oil) objectives. Gain and offset settings were kept constant for each antibody when imaging samples from the same experimental run. Bright-field images were also obtained for each sample. At least two sections per condition were imaged for each antibody. Images were taken at a resolution of 1024 × 1024 pixels.

Image analysis

Areas of fascicular matrix (FM) and interfascicular matrix (IFM) were identified visually based on appearance under bright-field settings, cell morphology and cell density. The FM, also known as the tendon proper, appears organized, consisting of highly aligned collagen fibers and cells with long, thin nuclei. The IFM, also known as the endotenon, is more disorganized in appearance, with a greater number of more rounded cells (Fig. 3). Blood vessels were also observed in some sections of the IFM. The percent area of FM and IFM in each image was calculated. All image analysis was performed using ImageJ (1.44, NIH).

COX-2 immunostaining appeared as bright, localized dots, often associated with the cell nuclei. For each image, the number of COX-2-positive cells was counted within the FM and IFM, and expressed per mm^2. Immunostaining for the other proteins of interest was more diffuse; therefore, the integrated pixel intensity of staining for each protein within the FM was calculated. To briefly detail this methodology, images were thresholded to remove background staining, using the same threshold value for all images, and integrated pixel intensity was calculated by summing the intensity of all pixels, and normalized to FM area. Several samples contained no IFM, so it was not possible to assess immuno-staining within the IFM in all loading conditions for all samples.

Statistical analysis

Differences in cell aspect ratio between control and loaded samples were assessed for statistical significance using a Mann-Whitney *U*-test as the data were non-normally distributed. Data are expressed as mean ± SEM.

Results

Cyclic loading to a constant peak strain resulted in a typical stress relaxation response (Fig. 1), with an average stress relaxation of 64.8 ± 16.2% after 1800 loading cycles.

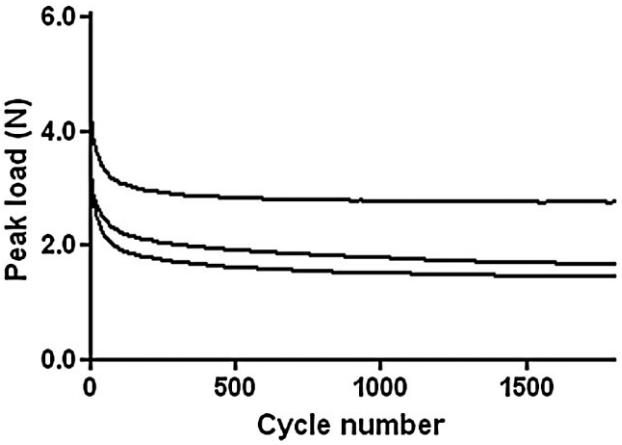

Fig. 1. Typical stress relaxation response of fascicle bundles from the SDFT. The peak load measured during each cycle is plotted against cycle number. At the end of 1800 loading cycles, peak load had decreased by an average of 64.8 ± 16.2%.

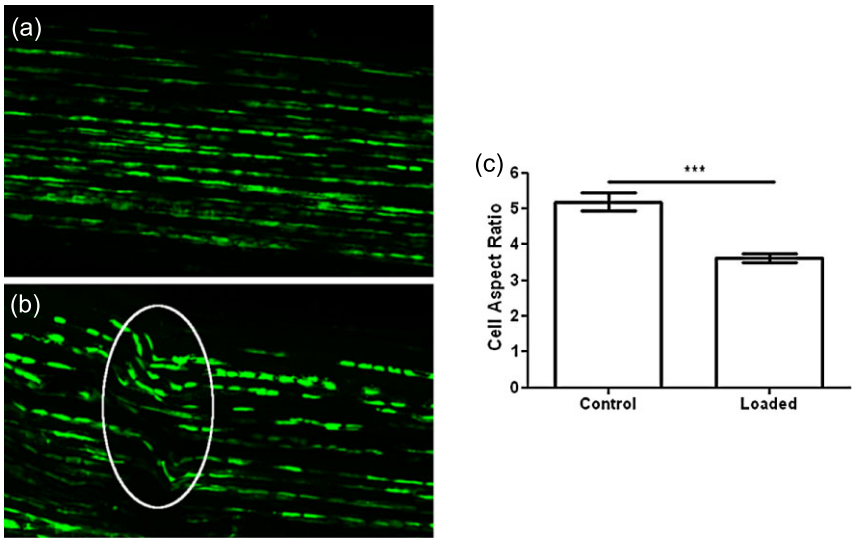

Fig. 2. Confocal images showing cells stained with calcein AM in control (a) and loaded samples (b). Cell aspect ratio decreased significantly ($P < 0.001$) after cyclic loading (c). Data are displayed as mean ± SEM. *** indicates significant difference ($P < 0.001$).

Cyclic loading results in alterations in cell morphology

In unloaded samples stained with calcein AM, cells within the fascicles appeared long and thin (Fig. 2(a)). Cell aspect ratio showed a significant decrease as a result of fatigue loading ($P < 0.001$, Fig. 2) with cells appearing rounder in loaded samples. This was particularly apparent in regions where damage, characterized by fiber kinking and change in cell alignment, could be observed (Fig. 2(b)).

Matrix degradation in the FM and IFM in fresh frozen controls

Control samples exhibited little or no positive staining for the inflammatory marker COX-2. All COX-2-positive cells identified in control samples were located within the IFM. Low levels of staining were observed for IL-6, MMP-3 and -13 in fresh frozen controls, with more intense staining for IL-6 and MMP-13 in the IFM. Greater staining for C1,2C and MMP-1 was also observed in the IFM, with co-localization of these proteins often observed (Fig. 3).

Effect of static load on markers of matrix inflammation and degradation

Exposing fascicle bundles to a very low static strain for 24 h resulted in decreased staining for IL-6 compared with fresh frozen controls (0.4-fold change, range: 0.3 to 0.5), and no apparent alterations in COX-2 levels, with no COX-2-positive cells identified in the FM of control or static samples (Fig. 4, left column). There was also no alteration in staining for MMP-1, -3, or -13, or C1,2C as a result of static strain (Figs 6 and 7, left column); therefore, these samples were used as the baseline to determine the fold changes in integrated pixel intensity, normalized to FM area, for markers of matrix inflammation and degradation following cyclic loading (Figs 4–7).

Effect of cyclic loading on matrix inflammation and degradation

Cyclic loading resulted in a more disordered matrix appearance, characterized by both fiber kinking and disruption of the IFM (Figs 4(d), 6(d), and 7(e)). It was not possible to assess changes in immunostaining within the IFM because of processing the artifact leading to absence of IFM in some sections. Loaded samples showed increased immunostaining for COX-2 and IL-6 in the FM (Fig. 4). The number of COX-2-positive cells increased from 0 cells/mm^2 in static controls to 284 ± 213 (mean ± 95% CI) cells/mm^2 in loaded samples. IL-6 staining was increased in the FM of all loaded samples, with a 4.0-fold increase (range 1.6 to 9.6) in integrated pixel intensity (Fig. 5).

Levels of MMP-3 showed no clear relationship with loading (Fig. 6), with staining only increasing with loading in the FM of one sample. Staining for MMP-13 increased within the FM of loaded samples, with a 2.3-fold increase (range 1.6 to 3.1) in integrated pixel intensity compared with static controls (Fig. 5).

Levels of MMP-1 showed no clear relationship with cyclic loading, with variable levels of staining between static and loaded groups (Fig. 7). Levels of C1,2C increased as a result of loading, with a 4.1-fold increase (range: 3.6 to 5.0) in staining in loaded compared with static samples (Figs 5 and 7). Colocalization of MMP-1 and C1,2C was observed in the majority of samples (Fig. 7).

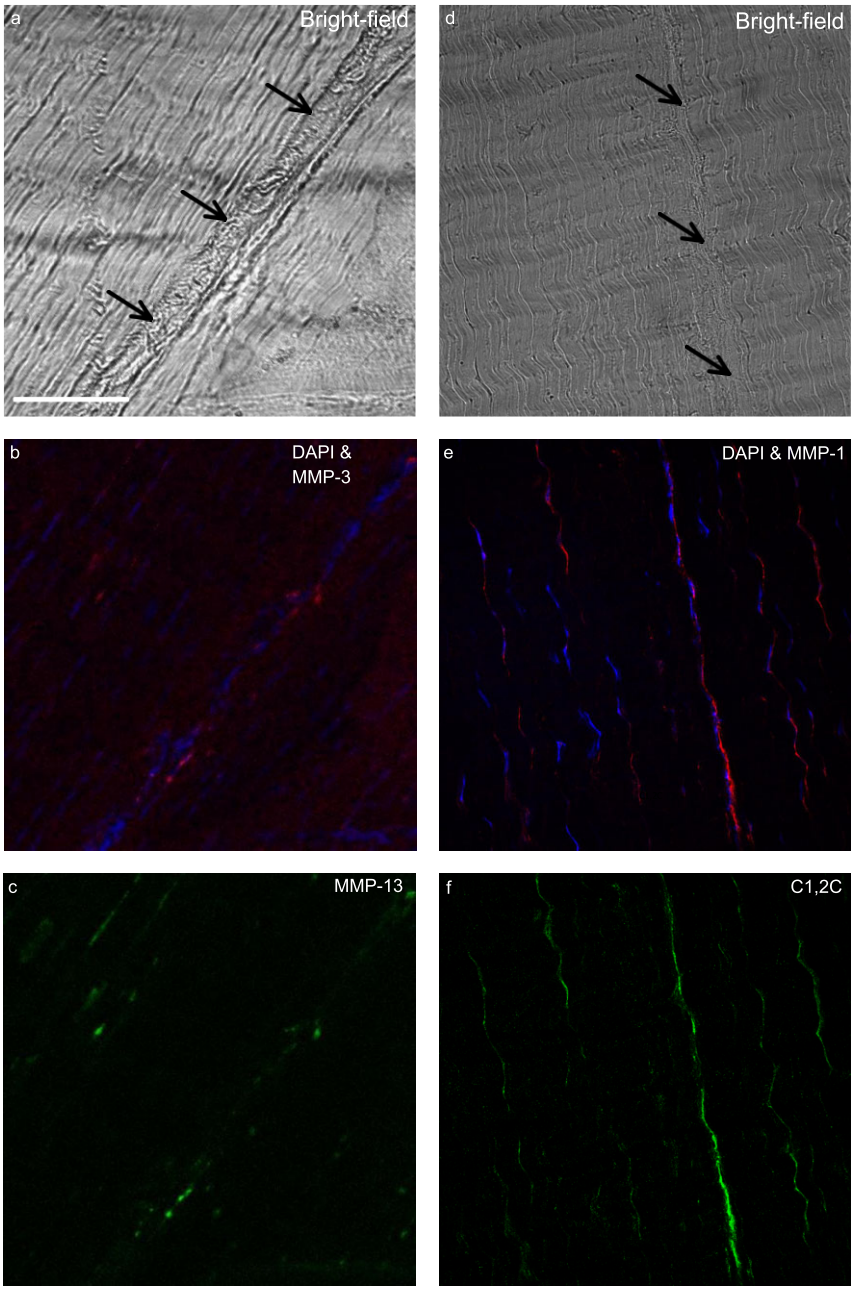

Fig. 3. Images from fresh frozen control samples showing bright-field images with IFM indicated by arrows (a, d), immuno-staining for cell nuclei (DAPI, blue) and MMP-3 (red, b), and MMP-13 (c). Immuno-staining for cell nuclei (DAPI, blue) and MMP-1 (red, e), and C1,2C (f). Scale bar = 50 μm. Note the greater staining for MMP-1 and -13, and C1,2C in the IFM compared with the FM.

Discussion

In this study, we have tested the hypothesis that cyclic fatigue loading of tendon explants increases matrix inflammation and degradation. The results support the hypothesis, demonstrating alterations in cell morphology, and increased levels of the inflammatory markers COX-2 and IL-6, as well as increased levels of MMP-13 and the collagen degradation marker C1,2C as a result of cyclic loading.

The cyclic loading protocol used in this study resulted in matrix damage as intended, characterized by both fiber kinking and disruption of the IFM, similar to that reported previously (Thorpe et al., 2014a, b). We have previously shown that fatigue loading results in alterations to SDFT fascicle microstructural strain response, and reduces the ability of fascicles to recover after loading (Thorpe et al., 2014a, b). In addition to these mechanical changes, the results of the present study show that loading also results in alterations to cell morphology, with cells within the FM becoming rounder. These changes are more pronounced in regions where damage to the matrix is evident. It is likely that in damaged regions, the cell strain environment would be

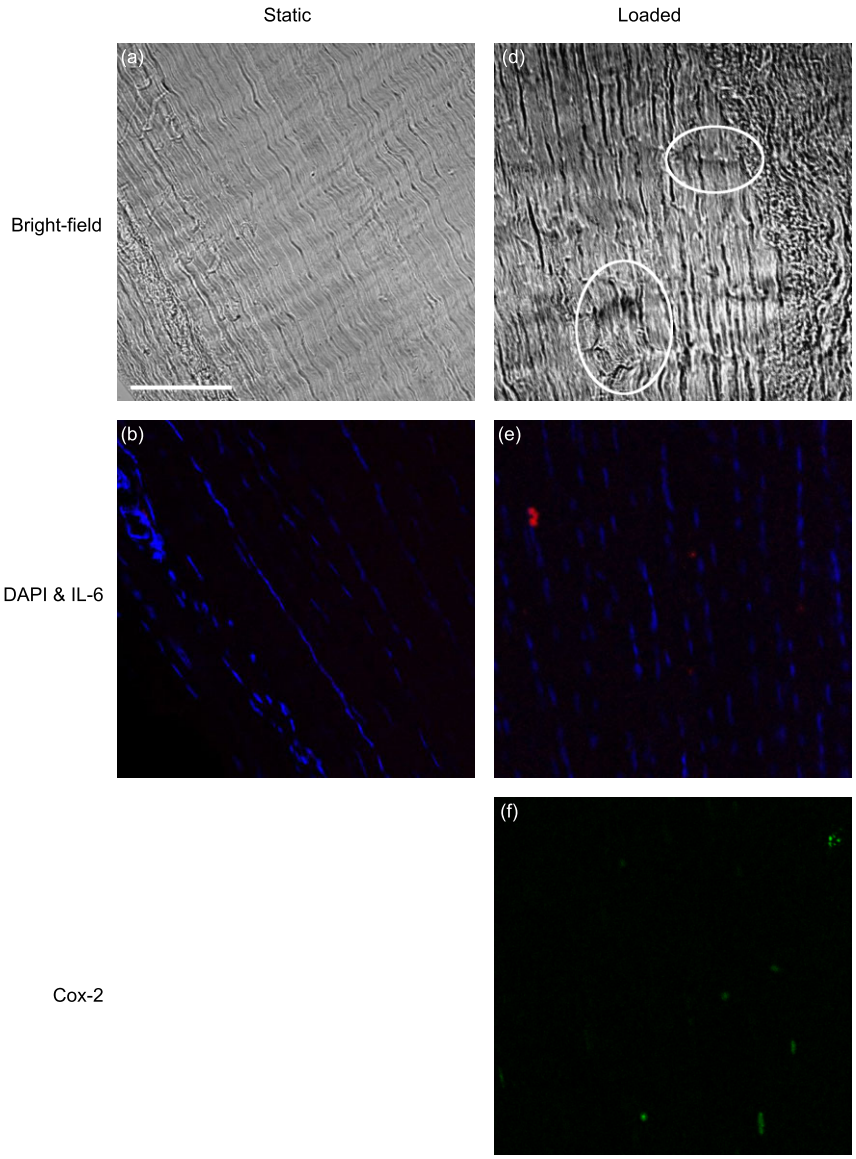

Fig. 4. Images from static (a–c) and loaded (d–f) samples showing bright-field images (a, d), immuno-staining for cell nuclei (DAPI, blue), and IL-6 (red; b, e), and Cox-2 (c, f). Scale bar = 50 μm. Areas of damage are circled in d.

altered and cell matrix interactions disrupted, which may cause the cells to round up. This is consistent with previous studies, which have reported the presence of round cell nuclei in tendinopathy and overloaded tendons (Scott et al., 2007; Riley, 2008) as well as in stress-deprived tendons (Arnoczky et al., 2008).

This change in cell shape was accompanied by increased numbers of COX-2-positive cells and increased staining for IL-6, indicating that tendon cells switch on inflammatory pathways in response to high levels of loading. This lends support to previous studies that have shown increased expression levels of IL-6 when loading fascicles *in vitro* (Legerlotz et al., 2013b) as well as in painful and ruptured Achilles tendons when compared with uninjured tendons (Legerlotz et al., 2012). However, the relationship between loading and IL-6 expression is complex, with a biphasic response reported previously, in which both stress-deprived and highly loaded samples from human hamstring tendons showed a larger increase in IL-6 expression than seen in samples subjected to low levels of loading (Legerlotz et al., 2013a). It has also been demonstrated that COX-2 mRNA and protein levels are upregulated in surgically ruptured tendon (Koshima et al., 2007; Bring et al., 2010), with higher levels of COX-2 mRNA in painful and ruptured Achilles tendons (Legerlotz et al., 2012).

While both COX-2 and IL-6 increased with fascicle loading, overall levels of staining for both inflammatory markers were low. This may be indicative of a low level inflammatory response, but it is possible that protein levels may peak earlier or later than the 24 h period studied in the current experiment. It has previously been shown that IL-6 protein levels in the peri-tendinous tissue peak immediately post-exercise, and then start to

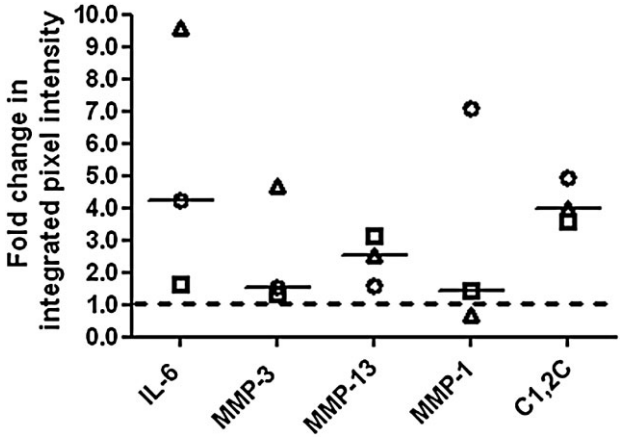

Fig. 5. Scatterplot showing fold changes in integrated pixel intensity normalized to FM area between static controls and loaded samples for IL-6, MMP-3, -13, and -1, and the collagen degradation marker C1,2C. Horizontal lines represent the median value. Data from individual tendons is represented by different symbols.

decrease (Langberg et al., 2002). Further, it is possible that the majority of the IL-6 produced diffused out of the tendon tissue and into the surrounding media and therefore would not be detected in this study. While no studies have investigated the temporal response of COX-2 immediately post-injury, a previous study was not able to detect any change in COX-2 3–6 weeks post-SDFT injury (Dakin et al., 2012a), suggesting that upregulation of these inflammatory pathways may be an immediate and transient response to overloading.

When considering changes in matrix-degrading enzymes as a result of loading, MMP-13 was the only MMP assessed that showed increased staining in loaded samples compared with static controls. This is in contrast to previous studies of painful and ruptured Achilles tendon, which showed downregulation of MMP-3, and no change in the expression of MMP-13 in painful or ruptured tendons, and upregulation of MMP-1 in ruptured tendons only (Jones et al., 2006). The MMP-1 upregulation observed by Jones et al. (2006) may be the result of an inflammatory response as inflammatory cells were observed in many of these samples, which were obtained 48 h post-rupture (Jones et al., 2006). In support of our findings, a previous study of natural SDFT injury in the horse demonstrated a 100-fold upregulation of MMP-13 in acute and chronic tendon injury (Clegg et al., 2007). A 10-fold upregulation of MMP-1 was reported in acute injury, with no change in chronic injury, whereas MMP-3 expression was downregulated in both acute and chronic injury (Clegg et al., 2007). Other studies that have assessed early changes as a result of loading have demonstrated increased MMP-13 expression in an *in vivo* rat patellar tendon model (Sun et al., 2008; Andarawis-Puri et al., 2012). *In vitro* studies have also reported alterations in MMPs in loaded fascicles, demonstrating an initial increase in MMP-3 and -13 expression immediately post-loading, followed by decreased expression 18–24 h after loading (Asundi & Rempel, 2008a; Maeda et al., 2009). Few studies have assessed the response of MMP-1 to loading, although it has been reported that MMP-1 is modulated by static, but not cyclic loading (Asundi & Rempel, 2008a, b).

Most studies that have investigated the early response to overload, both *in vivo* and *in vitro*, have assessed changes in mRNA levels, which may not translate to alterations at the protein level. However, in agreement with the results presented here, increased MMP-13 protein levels have been reported in overloaded rat tail tendon fascicles (Lavagnino et al., 2006), suggesting that this enzyme plays an important role in the initial response to matrix damage.

It is important to consider that MMP activity is tightly controlled at the protein level; MMPs within tendon may be inactive, either present in the pro form, or inhibited by tissue inhibitors of matrix metalloproteinases binding. Indeed, it has previously been demonstrated that activation of pro-collagenases is a key control point of collagen degradation in cartilage, and activation may occur days after stimulation of explants with pro-inflammatory mediators (Milner et al., 2001). Loading could therefore result in activation of MMPs via inflammatory pathways without overall changes in MMP protein levels. In addition, morphological changes to collagen fibrils induced by cyclic loading may render the collagen more susceptible to collagenase cleavage (Veres et al., 2014). These factors could account for the co-localization of MMP-1 with collagen degradation, but without increased staining for MMP-1 observed in the current study.

Several previous studies have assessed the tenocyte response to loading by straining isolated cells maintained in a 2D environment (Tsuzaki et al., 2003; Li et al., 2004). However, this is unlikely to be representative of the *in vivo* strain environment, in which cells are loaded predominantly in shear, with low levels of tensile strain compared with the overall applied strain (Screen & Evans, 2009; Thorpe et al., 2013). In the current study, tenocytes were maintained within their resident matrix, such that strains applied are likely to mimic those experienced *in vivo*. Twelve percent strain is equivalent to 60% of the fascicle bundle failure strain. This was designed to mimic the high levels of loading experienced during exercise; the equine SDFT may be exposed to strains up to 80% of its failure strain *in vivo* (Stephens et al., 1989; Thorpe et al., 2012), and we have previously shown that applying a similar strain regime results in visible microdamage within fascicles (Thorpe et al., 2014a, b).

The use of tissue explants, as used in this study, allows the tissue to remain viable by diffusion of nutrients during the loading protocol. However, we have previously found that cell death occurs at the cut surfaces of the explant. Testing of fascicle bundles as carried out in this study allowed the outer fascicles to be discarded following the loading regime and the fascicles within the

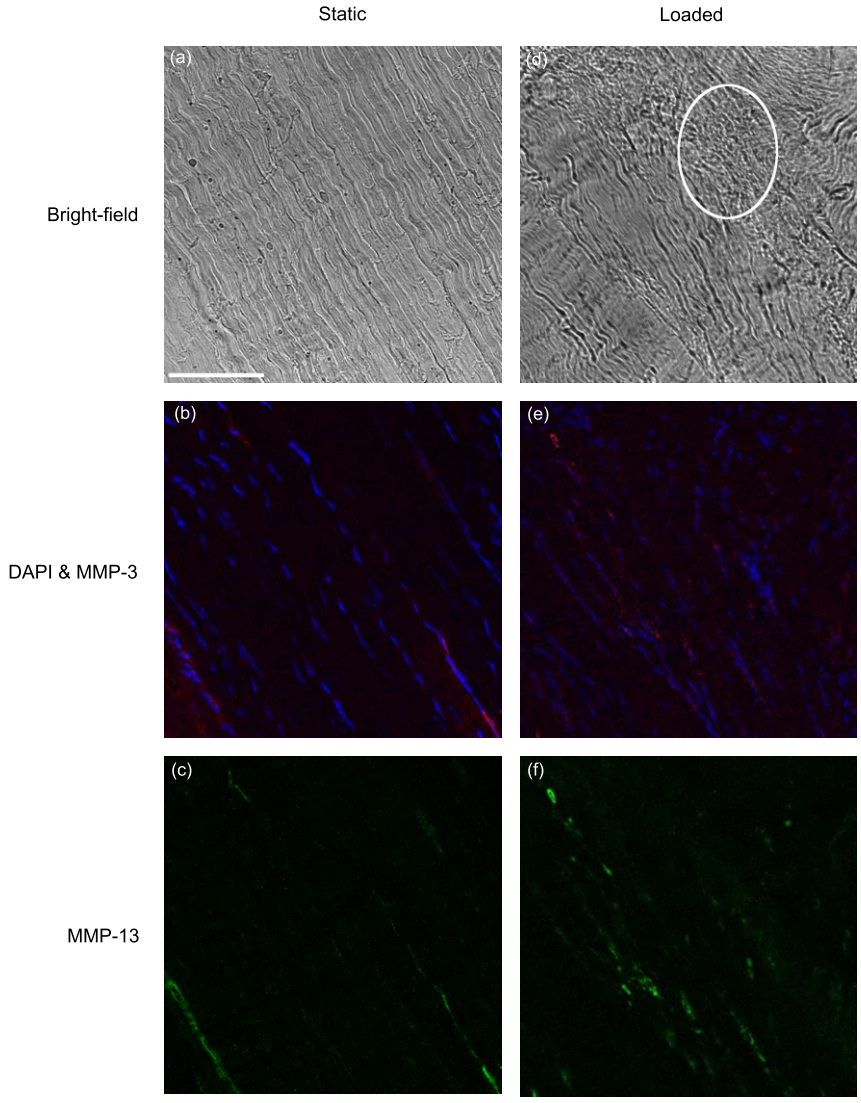

Fig. 6. Images from static (a–c) and loaded (d–f) samples showing bright-field images (a, d), immuno-staining for cell nuclei (DAPI, blue) and MMP-3 (red; b, e), and MMP-13 (c, f). Scale bar = 50 μm. Areas of matrix disruption are circled in d.

core of the sample, where cell viability was maintained, to be analyzed. Immunofluorescence allows visualization of damage and localization of proteins to specific compartments within the matrix. However, this cannot be considered a quantitative method, and as such, it is not possible to report statistically significant differences between experimental groups. Future studies are required to determine the size of the cell response to loading and to determine activation of MMPs.

Few previous studies have attempted to characterize phenotypic differences between the cells in the FM and IFM. It is evident that cell density and morphology differs within the IFM, with a greater number of more rounded cells than in the FM. Further, control samples showed greater staining for IL-6, MMP-1 and -3, and C1,2C within the IFM than in the FM. These results suggest that the cell population in the IFM is more active, maybe turning over this matrix more rapidly as shown in previous work where the noncollagenous component of tendon was found to have a higher turnover rate than the collagen (Thorpe et al., 2010). However, the loading response of the cells within this matrix remains unclear, as it was not possible to visualize the IFM in all loaded samples. It is of interest to note that the mechanical environment differs for cells within fascicles to those in the IFM. Previous work has shown that tendon extension results in sliding of fascicles such that the IFM may be subject to a high degree of shear loading *in vivo* (Thorpe et al., 2012). A possible source of inconsistency in this respect is that the sample test length used in the current study was short, such that the physiological IFM loading environment may not have been replicated.

The response of cells to fatigue damage in this study may be seen as a beneficial reaction. The results of this and previous studies suggest that pro-inflammatory cytokines may play an important role in healing during early stage injury. It has been shown that IL-6 induces the expression of MMP-9 via both COX-2-dependent

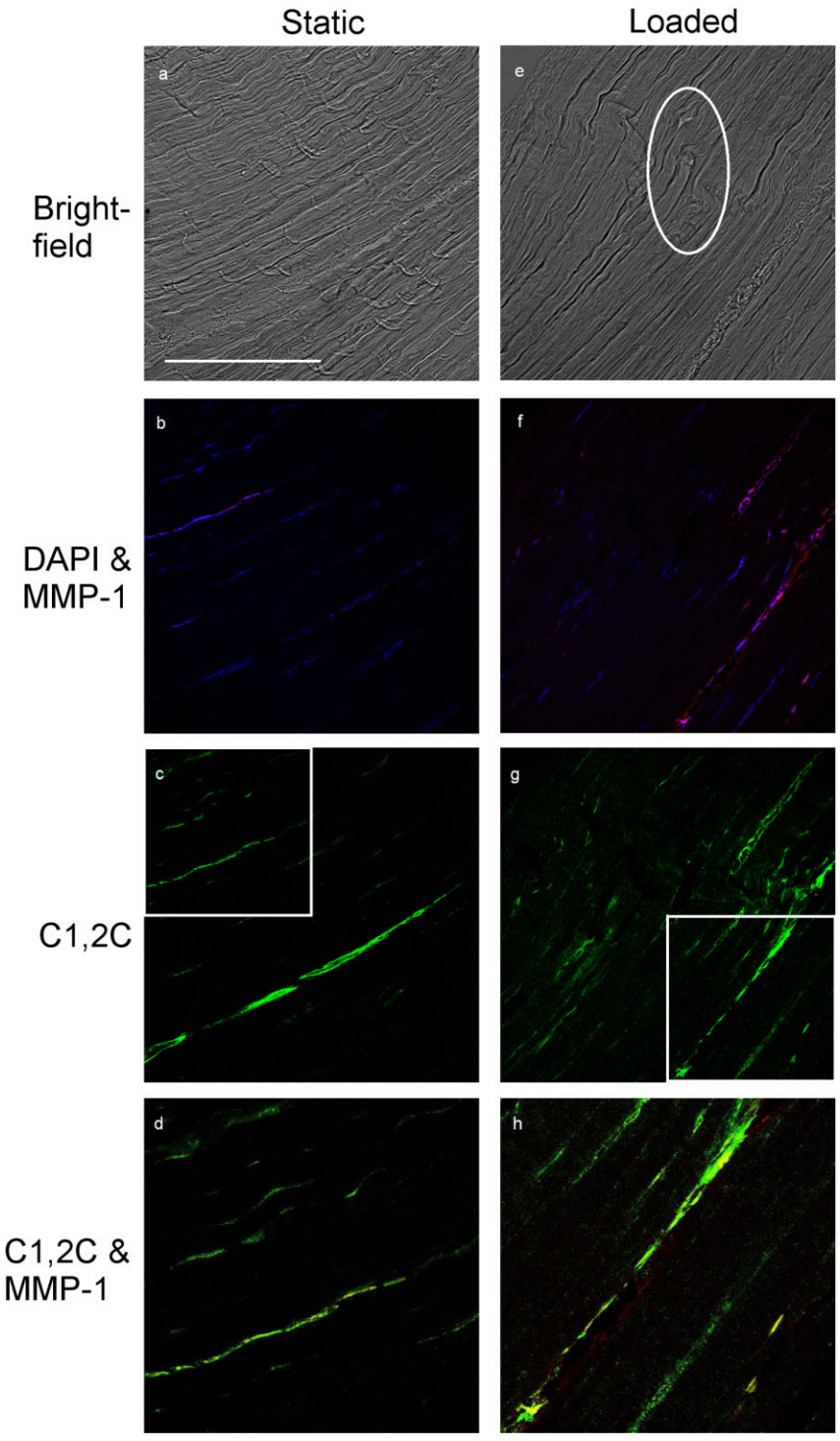

Fig. 7. Images from static (a–d) and loaded (e–h) samples showing bright-field images (a, e), immuno-staining for cell nuclei (DAPI, blue) and MMP-1 (red; b, f), and the collagen degradation marker C1,2C (c, g). Squares outlined in white in c and g are shown enlarged in d and h to demonstrate colocalization of MMP-1 and C1,2C (yellow). Scale bar = 50 μm. Areas of damage are circled in e.

and -independent pathways (Kothari et al., 2014), and treatment of cells with COX-2 inhibitors reduces the expression of MMP-13 and other MMPs (Asano et al., 2006). Further, IL-6 has been shown to induce collagen synthesis within tendon tissue (Andersen et al., 2011). The pathways by which inflammatory cytokines are activated are yet to be fully elucidated. However, our data suggest that the response may be driven by the cell response to an altered local strain environment because of localized microdamage within the tendon tissue. It is clear that cell shape changes occur in damaged regions, and this may initiate a signaling cascade, resulting in the production of enzymes to degrade damaged regions. Increased MMP production is likely important for

degradation of damaged regions of the tendon matrix. An initial inflammatory response may therefore be beneficial for healing of the injury; however, if this inflammation is not resolved, it may lead to an impaired healing response, with production of fibrotic tissue (Dakin et al., 2014). It is not clear why, in some injuries, inflammation becomes chronic, although it has been demonstrated that aged tendons have a reduced capacity to resolve inflammation (Dakin et al., 2012a). A fuller understanding of these pathways will enable the development of techniques to improve the healing response of tendon tissue.

Cyclic loading of equine tendon explants *in vitro* resulted in damage to the tendon matrix, causing alterations in cell morphology, likely because of altered cell strain environment and cell matrix interactions. These alterations in cell shape are accompanied by increased levels of pro-inflammatory mediators and matrix degradation. The presence of an inflammatory component in the cell response to matrix damage is likely to be important to the tendon healing response. Full understanding of the early tendon cell response to microdamage is important for developing effective treatment options for tendon injury.

Perspectives

Tendon injury is common, with a poor prognosis in many individuals and few effective treatment options. This is one of the first studies to investigate the early cell response to overloading *in situ*, demonstrating increased inflammation and matrix degradation in loaded samples. This provides important information regarding the initiation of tendinopathy, suggesting that inflammation may play an important role in the initial cell response to tendon damage. Full understanding of the early tenocyte response to matrix damage is critical in order to develop effective treatments for tendinopathy.

Key words: Tendinopathy, metabolism, tenocyte, fatigue, microdamage, collagen, fascicle, interfascicular matrix.

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Supporting information

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

Fig. S1. Western blotting validation of antibodies for MMP-13 (a) and MMP-1 (b).