IN VIVO BIOLOGICAL RESPONSE TO EXTRACORPOREAL SHOCKWAVE THERAPY IN HUMAN TENDINOPATHY

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Abstract

Extracorporeal shock wave therapy (ESWT) is a non-invasive treatment for chronic tendinopathies, however little is known about the *in-vivo* biological mechanisms of ESWT. Using microdialysis, we examined the real-time biological response of healthy and pathological tendons to ESWT. A single session of ESWT was administered to the mid-portion of the Achilles tendon in thirteen healthy individuals (aged 25.7 ± 7.0 years) and patellar or Achilles tendon of six patients with tendinopathies (aged 39.0 ± 14.9 years). Dialysate samples from the surrounding peri-tendon were collected before and immediately after ESWT. Interleukins (IL)-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-17A, vascular endothelial growth factor and interferon-γ were quantified using a cytometric bead array while gelatinase activity (MMP-2 and -9) was examined using zymography. There were no statistical differences between the biological tissue response to ESWT in healthy and pathological tendons. IL-1β, IL-2, IL-6 and IL-8 were the cytokines predominantly detected in the tendon dialysate. IL-1β and IL-2 did not change significantly with ESWT. IL-6 and IL-8 concentrations were elevated immediately after ESWT and remained significantly elevated for four hours post-ESWT ($p < 0.001$). Pro-forms of MMP-2 and -9 also increased after ESWT ($p < 0.003$), whereas there were no significant changes in active MMP forms. In addition, the biological response to ESWT treatment could be differentiated between possible responders and non-responders based on a minimum 5-fold increase in any inflammatory marker or MMP from pre- to post-ESWT. Our findings provide novel evidence of the biological mechanisms underpinning ESWT in humans *in vivo*. They suggest that the mechanical stimulus provided by ESWT might aid tendon remodelling in tendinopathy by promoting the inflammatory and catabolic processes that are associated with removing damaged matrix constituents. The non-response of some individuals may help to explain why ESWT does not improve symptoms in all patients and provides a potential focus for future research.

Keywords: Microdialysis, interleukin, mid-portion tendinopathy, conservative treatment, matrix metalloproteinase, extracorporeal shock wave therapy.

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Introduction

Tendons are highly organised, hierarchical structures that transmit muscular forces to the skeletal system for creating movement or for joint stability. For effective force transfer, tendons are required to be stiff, whilst some extensibility allows energy storage for efficient locomotion (Shepherd and Screen, 2013). Tendons must be able to withstand the forces generated through muscular loading. However, repetitive and/or excessive overloading of the tendon, such as that experienced with endurance running or poor biomechanics is considered to be the major aetiological factor in the development of tendinopathies (Huang *et al.*, 2004; Kader *et al.*, 2002). Tendinopathies are a common and painful problem affecting both athletes (Kvist, 1991) and sedentary individuals (Rolf and Movin, 1997) causing a loss of function. Pathological changes, such as collagen fibre and fibril disorganisation, neovascularisation, and an increase in tenocyte proliferation and non-collagenous matrix quantity all provide evidence of a failed healing response to accumulated tendon damage (Astrom and Rausing, 1995; Jozsa and Kannus, 1997).

There are a number of conservative treatment options which have been shown to improve the symptoms associated with tendinopathy, including physiotherapy, high-volume image-guided injections and eccentric loading (Coombes *et al.*, 2010; de Vos *et al.*, 2010; Sussmilch-Leitch *et al.*, 2012). Whilst some papers have questioned its efficacy (Costa *et al.*, 2005; Zwerver *et al.*, 2011), a recent systematic review demonstrated that extracorporeal shock wave therapy (ESWT) is also largely successful in providing an alternative conservative tendinopathy treatment (10/11 papers examining the efficacy of ESWT in Achilles tendinopathy, and 6/7 papers for the patellar tendon report a significant improvement in symptoms after treatment; Mani-Babu *et al.*, 2014). ESWT is a non-invasive treatment in which the kinetic energy of acoustic shockwaves is concentrated on the area of pain and pathology, therefore applying mechanical forces to the tissue. Shockwaves are pressure waves, characterised by a rapid rise in positive pressure and high peak pressure amplitude, followed by a fall in pressure below ambient, *i.e.* negative pressure. The waves augment tissue density as a result of the positive and negative phases of the propagating wave passing through it, and in doing so deliver direct mechanical perturbations to the tissue. In the low-pressure phase of a passing shockwave, ESWT also causes cavitation (Ogden *et al.*, 2001), characterised by the formation of gas bubbles. The implosion of the bubbles with the rise in pressure from the subsequent shock wave produces a secondary energy wave, which also provides a mechanical stimulus (Delius, 1994; Schmitz *et al.*, 2013).

The mechanical perturbations generated by ESWT are likely to be of key importance to its treatment effect; due to the mechanosensitive nature of tendon cells, the impact of ESWT on interstitial and extracellular processes are theorised to initiate tissue regeneration (Ogden *et al*., 2001).

In vitro, using explant models and cell culture techniques, ESWT has been shown to enhance angiogenesis (Chen *et al.*, 2004; Wang *et al.*, 2003), and increase tenocyte proliferation, collagen synthesis (Vetrano *et al.*, 2011), glycosaminoglycan (GAG) content, protein synthesis (Bosch *et al.*, 2009; Bosch *et al.*, 2007) and growth factors – such as transforming growth factor (TFG)-β1 – known to regulate tendon repair (Chen *et al*., 2004). It has also acted to decrease the presence of inflammatory cytokines (Han *et al.*, 2009). Although insightful, such approaches have significant limitations, as the physiological environment of the tissue is not maintained, and thus it is difficult to relate findings to the *in vivo* response. *In situ* studies, which maintain the tissue's physiological environment, have proven useful in assessing long-term structural changes resulting from ESWT treatment, such as potential reductions in tendon thickness (Wang *et al.*, 2007). However, they provide no information regarding the biological environment of the tissue, or the immediate biological response of the tissue to the treatment. To our knowledge, little is known about the biological effect of ESWT on tendon tissue *in vivo*. Therefore, the mechanisms associated with the positive effect of ESWT on Achilles tendinopathy are currently unclear.

Microdialysis can be used to examine the real-time biology of soft tissue intercellular spaces (Lonnroth *et al.*, 1987) and has been used extensively in recent years to enrich our understanding of matrix turnover and metabolic processes occurring at a local tendon level at rest (Andersen *et al.*, 2011), in response to mechanical loading (Koskinen *et al.*, 2004; Langberg *et al.*, 2002b), with injury (Alfredson *et al.*, 2002), and during injury repair processes (Ackermann *et al.*, 2013; Greve *et al.*, 2012). The aim of this study was to examine the metabolic response of normal and tendinopathic tendons to ESWT treatment and provide the first *in vivo* evidence of the biological mechanisms underpinning ESWT treatment outcomes. Specifically, we investigated the acute response of inflammatory cytokines associated with mechanical loading, repair processes and tenocyte health (John *et al.*, 2010; Thorpe *et al.*, 2015), and also matrix metalloproteases (MMPs), which are implicated in the homeostasis of tissue regeneration (Jones *et al.*, 2013; Riley *et al.*, 2002), for the purpose of improving our understanding of the mechanisms underlying observed effects.

Materials and Methods

Ethical approval and participant information

Institutional ethical approval was granted by the Queen Mary University of London ethics of research committee. The research was conducted in accordance with the Declaration of Helsinki guidelines. Thirteen participants with healthy tendons (herein referred to as 'healthy participants'; 7 men, 6 women, aged 25.7 ± 7.0 years) were recruited from the university and general public by local and online advertisement. For inclusion into the study, healthy participants must not have had a previous tendinopathy or other tendon-related disorder (*e.g.* Haglund's deformity or patello-femoral pain). Six patient participants (6 men, aged 39.0 ± 14.9 years) were recruited using leaflets displayed by local orthopaedic clinics and using an online advertisement published on a national running website. For inclusion into the study, patient participants were required to have an established (*i.e.* symptomatic for > 6 months) uni- or bi-lateral patellar or mid-portion Achilles tendinopathy, diagnosed by a suitably qualified healthcare professional. Patients were excluded from participation if they had received steroid, platelet-rich plasma or high-volume image-guided injections in the previous 6 months, or had previous tendon surgery.

All participants were required to be aged between 18 and 65 years at the time of data collection and were screened for a history of systemic inflammatory conditions, anti-inflammatory medication prescriptions, and known allergy to or conditions contradicting the use of Lidocaine. All participants provided fully informed written consent and patient participants additionally completed a condition severity questionnaire (VISA-A or VISA-P for Achilles or patellar tendinopathy, respectively (Robinson *et al.*, 2001; Visentini *et al.*, 1998)) as a means of assessing pain and function. Data were collected from both tendons in some healthy participants, resulting in a total of 19 samples from the 13 healthy participants. For patient participants, data was collected from clinically pathological tendons only. Four patients had bilateral tendinopathy, leading to a total of 10 samples from the 6 patient participants. Patient and healthy participant demographics are presented in Table 1.

Table 1. Demographic information relating to healthy and patient groups.

Group	Subject #	Age	Tendon	Tendon #	VISA score	IL	MMP
Healthy	1-8	24.4 ± 1.6	Achilles	10	-	√	√
	9-13	27.2 ± 9.9	Achilles	9	-	x	√
Patient	1-3	51.4 ± 8.7	Achilles	4	61.3 ± 8.3	√	√
	4-6	26.7 ± 3.9	Patellar	6	41.0 ± 11.5	√	√

[1]A number of healthy participants consented to sampling from both tendons, and some patient participants had bilateral tendinopathy, so both number of participants and number of sample tendons are indicated for each group.

[2]VISA-A or VISA-P scores are provided for Achilles and patellar tendinopathy participants, respectively.

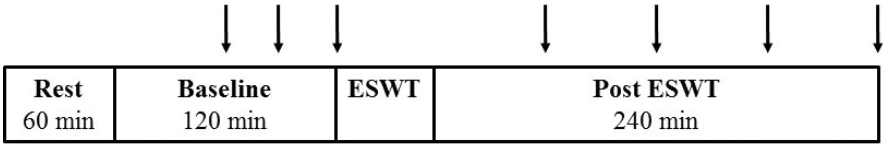

Fig. 1. Schematic of protocol. Black arrows represent sampling time points.

Experimental protocol

Participants were instructed not to ingest anti-inflammatory drugs or partake in heavy exercise over the 48 h period prior to testing. All experiments started at approximately 09.00 hours, and are shown schematically in Fig. 1. Participants lay prone (Achilles) or were seated (patellar) while the microdialysis probe was inserted. Due to a possible immediate biological response of the tissue to the trauma of the microdialysis probe insertion (Langberg *et al.*, 1999), a 60 min wait period was introduced prior to commencing dialysate collection. From this point, dialysate was continually collected, and pooled over 30 min intervals to provide sufficient sample for analysis. Dialysate from 60-90 min provided the baseline samples for MMP analysis; dialysate from 90-120 min provided the baseline sample for cytokine analysis. ESWT was administered to each tendon mid-portion (mean treatment size of 1-2 cm^2) in a single session using a British kite-marked device (Swiss DolorClast® Classic, Electro Medical Systems, Nyon, Switzerland) and radial hand piece. Treatment was given perpendicular to the longitudinal tendon axis without anaesthesia and consisted of 2500 impulses administered at 8 Hz. The total energy delivered was 160 mJ/mm. After ESWT treatment, dialysate was collected every 60 min for the following 4 h. Achilles participants remained prone with the ankle joints in a relaxed neutral position (70-80°) throughout dialysate collection. For effective ESWT administration, the ankle was briefly moved to ~85°**.** Patellar participants sat in Fowler's position with a minimally flexed, supported knee (160-170°), for both dialysate collection and ESWT administration.

Microdialysis

The details of the procedure were based on the technique as described by Lonroth *et al*. (1987). Positioning the microdialysis catheters (Langberg *et al.*, 2001) entailed a bilateral procedure under strict aseptic technique. For healthy participants, the skin either side of the free Achilles tendon was anaesthetised using subcutaneously injected Lidocaine (0.4-0.7 mL, 20 mg/mL), approximately 25 mm proximal to the calcaneal bone. For patient participants, the skin either side of the site with most pain and/or tendon thickening on palpation, depending on proximity to bony structures, was anaesthetised irrespective of the tendon type. Under ultrasound guidance (Voluson-I, GE Medical, Zipf, Austria), the active part of the microdialysis catheter was positioned in the peritendinous space (ventral to the Achilles tendon and superior to the patellar tendon). A high-precision fixed rate infusion pump (CMA 402, CMA Microdialysis AB, Kista, Sweden) perfused the catheter with sterile lactated Ringer's solution at a rate of 2 µL/min (Langberg *et al*., 2001). The dialysate fluid obtained from the probe outlet was collected in a centrifuge tube connected to the outlet tubing. Dialysate samples were immediately stored at -80 °C until further analysis.

Dialysate analysis

The profiles of interleukin (IL)-1β, IL-2, IL-4, IL-6, IL-8, IL-10, IL-12p70, IL-17A, vascular endothelial growth factor (VEGF) and interferon (IFN)-γ were quantified by a cytometric bead array (healthy – human flex set, patient – human enhanced sensitivity flex set; BD Biosciences Pharmingen, San Diego, CA, USA). Samples were diluted 1:2 (vol/vol) before analysis. Briefly, samples and standards were incubated first with capture beads, and then detection reagents, to form sandwich complexes for each detectable cytokine. The complex formed for each cytokine could be differentiated based on its unique fluorescence characteristics, which were identified and quantified using flow cytometry (Accuri™ C6 and CFlow software, BD Biosciences Pharmingen).

Gelatin zymography was used to quantitate the relative amounts of MMP-2 and MMP-9 activity at baseline, and also 1, 2 and 4 h after ESWT treatment. Zymography was performed using a 10 % sodium dodecyl sulphate (SDS) polyacrylamide gel containing 1 mg/mL gelatin, overlain with 5 % stacking gel. Samples were mixed 3:1 (vol/vol) with non-reducing loading buffer (200 mM Tris pH 6.8, 4 % SDS, 0.1 % Bromophenol blue, 40 % glycerol) and 20 µL loaded in each well. Electrophoresis was carried out at 150 V until the dye front had reached the bottom of the gel. Gels were rinsed twice in 2.5 % Triton X-100 and incubated in a development buffer (50 mM Tris pH 7.5, 5 mM $CaCl_2$) at 37 °C for 36 h. Gelatinase activity was revealed by negative staining with Coomassie Brilliant Blue. MMP-2 and MMP-9 standards (R&D Systems Europe Ltd, Abingdon, UK) were used as positive controls for gelatinase activity identification, and precision plus protein™ standard (Bio-Rad Laboratories, Hercules, CA, USA) was run on each gel to provide approximate protein sizes. 0.1 mM Ethylenediaminetetraacetic acid (EDTA) was added to the loading buffer, and 1 mM EDTA added to the development and rinse buffers of duplicate gels, to inhibit MMP activity on samples loaded onto the second gel, thus acting as a control. Gels were scanned with a Li-Cor Odyssey scanner (Lincoln, NE, USA). Images were inverted and semi-quantitative densitometry performed using Image-J (NIH, Bethesda, MD, USA).

Statistical analysis

Data analysis was conducted with IBM SPSS version 20.0 (IBM Corp, Armonk, NY, USA). All data are presented as

means ± standard error of the mean unless otherwise stated. Statistical analyses relating to the examination of cytokines were performed on data collected from 10 patient tendons and the first 10 healthy participant tendons that data were collected from (Table 1). For the purposes of calculating mean concentrations and performing statistical tests on the generated data set, analyte concentrations from dialysate samples found to be lower than the detection limit were set to the theoretical detection limit as described in the flex set data sheet (Ackermann *et al*., 2013). To allow a comparison of analyte concentrations between groups, the detection limits for the standard flex set were adopted for all samples. Detection thresholds for each cytokine were as follows: IL-1, 1,260 fg/mL; IL-2, 210 fg/mL; IL-4, 7,290 fg/mL; IL-6, 970 fg/mL; IL-8, 920 fg/mL; IL-10, 780 fg/mL; IL-12, 9,600 fg/mL; IL-17, 11,350 fg/mL; IFN-γ, 9,300 fg/mL and VEGF, 2,200 fg/mL. Cytokine concentration data were log-transformed to control for heteroscedasticity. Baseline cytokine concentrations were compared between healthy and patient tendons using independent *t*-tests. Two-way analyses of variance (ANOVA) with repeated measures were used to examine each cytokine with respect to time (pre-ESWT and 1, 2, 3, 4 h post-ESWT) and group (healthy, patient). In the case of significance, a one-way ANOVA was performed for each dependent variable, followed by Bonferroni *post hoc* tests to identify the location of the significant differences between groups. Statistical significance was accepted at $p < 0.05$.

Statistical analyses relating to the examination of MMP activity were performed for data collected from 10 patient tendons and 19 healthy participant tendons (Table 1). Protein bands were identified as zymographic activity by comparing to the positive control standards and protein size ladder on each gel. To compare baseline MMP activity between groups, the relative density (*i.e.* intensity) of each protein band expressed at baseline was calculated as the fold-change in density from that of the 9 µL MMP-2 and MMP-9 standards (1 ng/µL) run on each gel (a relative density of 1). Baseline MMP activity was compared between groups using a Mann-Whitney U test. The relative density of each protein band relating to a single data set was then calculated as the fold-change in density from that of the baseline sample. Friedman test for non-parametric data was used to compare each MMP before and 1, 2 and 4 h after ESWT for each group. In the case of significance, Wilcoxon's signed rank tests were used to identify where differences were located. Statistical significance was accepted at $p < 0.05$.

Results

Inflammatory cytokines

IL-1β, IL-2, IL-6 and IL-8 were detected in both healthy and patient tendons. Low levels of IL-10 and IFN-γ were detected in some patient tendons (40 % and 30 % detection rate, respectively), but not in healthy tendons; all other cytokines investigated were below detection levels or not present in the dialysate. Due to low detection rates of IL-10 and IFN-γ, statistical analysis was performed on IL-1β, IL-2, IL-6 and IL-8 only. Independent *t*-tests performed on baseline cytokine concentrations demonstrated a significant lower concentration of IL-2 in patients than in healthy tendons pre-ESWT ($p = 0.005$); there was no significant difference in baseline concentrations of IL-1, -6 or -8 ($p = 0.123$-0.208, Fig. 2a). Results of the repeated measures ANOVAs established that there were no time-by-group interactions for any cytokine examined. There were main

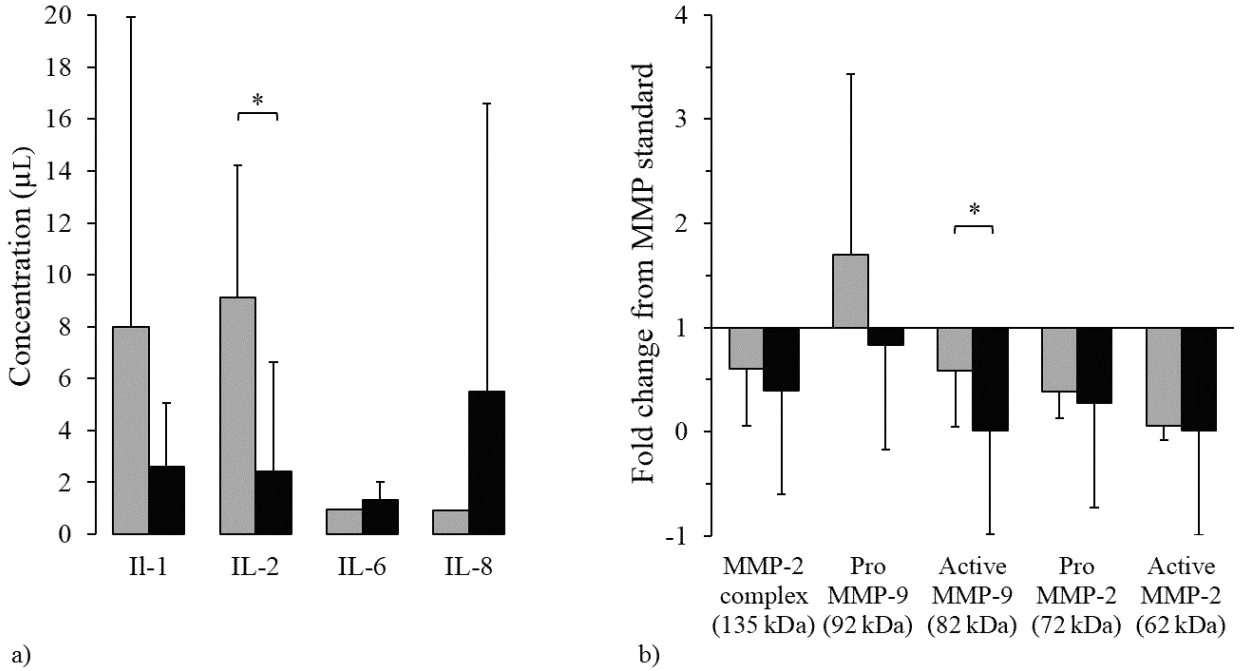

Fig. 2. a) Mean (+SD) concentration of interleukin (IL)-1β, 2, 6 and 8 exhibited by healthy (grey bars, $n = 10$) and patient (black bars, $n = 10$) tendons at baseline, and **b**) MMP expression exhibited by healthy (grey bars, $n = 18$) and patient (black bars, $n = 10$) tendons at baseline. MMP expression was calculated relative to the densities of 9 µL MMP-2 and -9 standards (1 ng/µL) run on each gel, determined using densitometry. * $p < 0.05$.

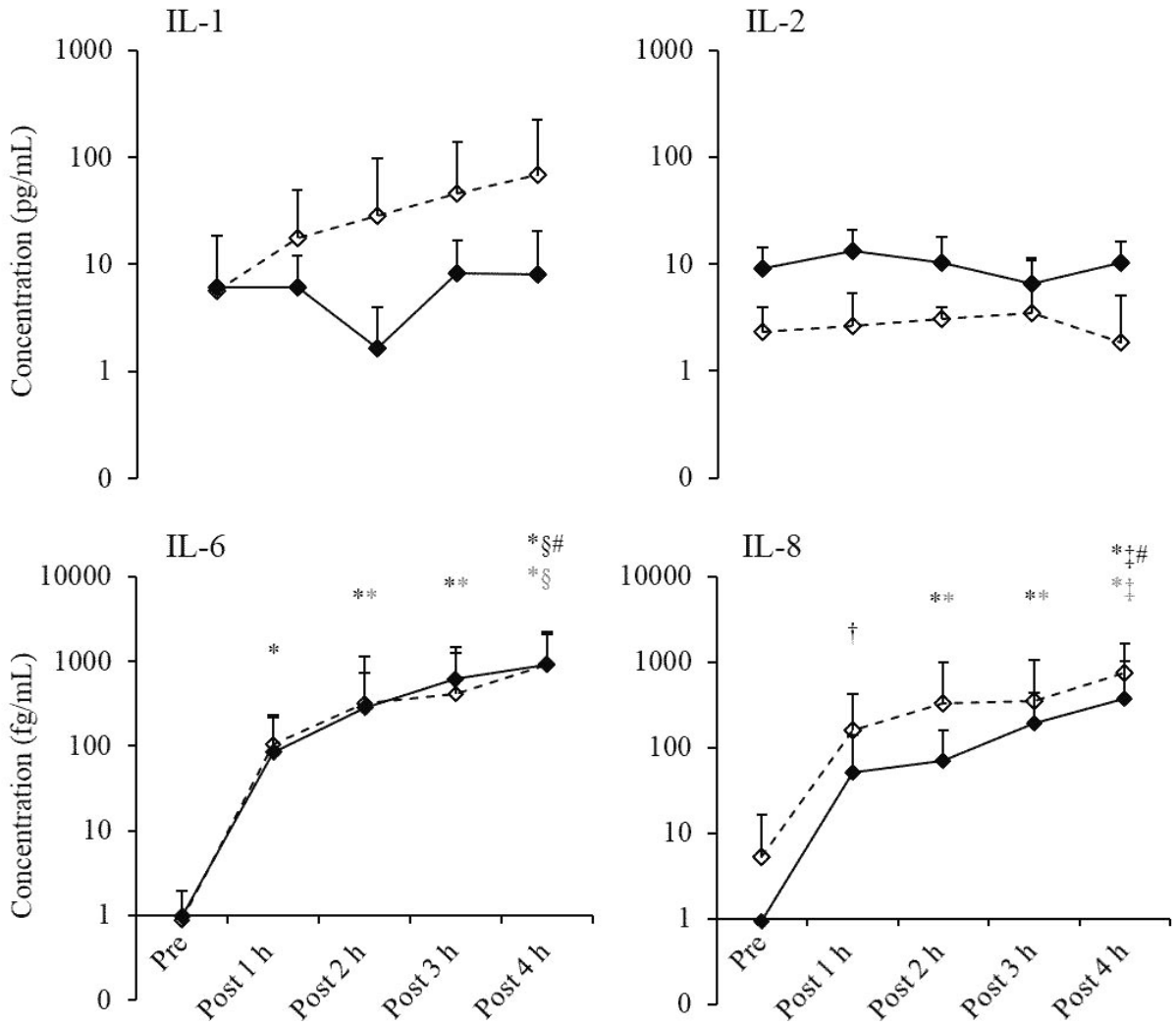

Fig. 3. Concentration of interleukin (IL)-1β, 2, 6 and 8 before and 1, 2, 3 and 4 h after ESWT in healthy (filled diamonds, $n = 10$) and tendinopathic (open diamonds, $n = 10$) tendons. IL-6 and IL-8 demonstrate significantly elevated concentrations post-ESWT when compared with pre-treatment concentrations and remained significantly elevated 4 h post-ESWT. * Different from baseline value ($p < 0.05$); † different from baseline ($p < 0.1$); § different from post 1 h ($p < 0.05$); ‡ different from post 1 h ($p < 0.1$); # different from post 2 h ($p < 0.05$). Black and grey symbols refer to patients and healthy groups, respectively.

effects of time for IL-1β ($F(1,18) = 2.754$, MSE = 0.429, $p = 0.034$), IL-6 ($F(1,18) = 29.745$, MSE = 20.116, $p < 0.001$) and IL-8 ($F(1,18) = 24.990$, MSE = 14.653, $p < 0.001$), but not for IL-2 ($F(1,18) = 1.005$, MSE = 0.185, $p = 0.411$). The individual ANOVA results demonstrated elevated levels of IL-6 and IL-8 post-ESWT for both healthy ($p = 0.001$) and patient tendons ($p = 0.001$) and concentrations remained significantly elevated 4 h post-ESWT (Fig. 3). The main effect of time for IL-1β was not demonstrated when groups were analysed separately.

Matrix metalloproteinase activity

MMP-2 activity was detected at ~135, 72 and 62 kDa. MMP-9 activity was detected at 92 and 82 kDa (Fig. 4). EDTA inhibited all MMP activity, confirming that bands seen on the zymogram were a result of MMP activity. Pro-MMP forms were more strongly detected than active forms. Densitometry data from baseline dialysate samples demonstrated significantly lower gelatinase activity of MMP-9 (82 kDa) in the patient group ($U = 24$, $p = 0.001$) pre-ESWT; there were no differences in MMP activity at any other molecular weight between groups (Fig. 2b). MMP-2 complex (135 kDa) and pro-MMP-9 (92 kDa) activity were significantly elevated post-ESWT relative to baseline in both healthy and patient groups (135 kDa, $\chi2(3) = 14.200$, $p = 0.003$; 92 kDa, $\chi2(3) = 17.325$, $p = 0.001$), whereas pro-MMP-2, and active MMP-2 and -9 did not change significantly at any time point after ESWT (Fig. 5). The large within-group variability in MMP data, which may be responsible for the lack of detectable changes at certain time points, appears to be a result of a response or non-response to ESWT. Responders were defined as exhibiting a minimum 5-fold increase in any MMP at any time point post-ESWT when compared to baseline values (response rate of 22 % and 60 % in healthy and patient tendons, respectively; Fig. 6).

Discussion

The present study demonstrates an immediate and significant increase in some inflammatory and metabolic markers in response to ESWT in both healthy and pathological tendons. To our knowledge, these findings are the first to document the immediate *in vivo*, biological response of both healthy and pathological tendons to ESWT, and provide a novel insight into the mechanisms underpinning the effect of ESWT when used as a treatment for non-insertional tendinopathies, showing distinct molecular responders and non-responders based on a single session of ESWT.

Inflammatory cytokines

Inflammatory cytokines IL-1β, IL-2, IL-6 and IL-8 were detected in both healthy and patient tendons. No other cytokines were detected in healthy tendons, whilst low levels of IL-10 and IFN-γ were detected in some patient tendons. These resting cytokine profiles are largely in keeping with those reported by Ackermann *et al*. (2013) for healthy tendons, although their study reported a mean concentration of 2.5 pg/mL for IL-10 in healthy tendons which is significantly greater than the detection threshold for IL-10 in the current study (0.78 pg/mL). Cytokines can have a potent effect, even at picomolar concentrations (Dakin *et al.*, 2014). Although this may represent a genuine difference between the participants recruited to each study, Ackermann *et al*. used the contralateral tendon of patients with uni-lateral Achilles tendinopathy as their healthy tendon comparison, so may have seen a contralateral effect, due to the study's within-subject design (Andersson *et al.*, 2011).

Resting levels of IL-2 were significantly lower in patient tendons than healthy tendons in the current study, similar to the down-regulation of IL-2 seen in degenerative rat supraspinatus tendons (Millar *et al.*, 2009). IL-2 is a pro-inflammatory mediator, produced and stored by T-cell lymphocytes. Although there is little other evidence of the direct involvement of IL-2 in tendinopathy, T-cells are important in the onset of several inflammatory diseases. Despite the on-going debate regarding inflammation in tendinopathy (Rees *et al.*, 2014), we might speculate that lower levels of IL-2 in chronic tendinopathy patients reflects a reduced T-cell presence in comparison to healthy tendons – although there is evidence to suggest the opposite may be true (Schubert *et al.*, 2005). There were no differences in the concentrations of IL-1, -6 or -8 between groups at baseline. This finding is not altogether surprising given the lack of consensus from published literature on the topic. For example, IL-1β may be increased (Gotoh *et al.*, 1997; Hosaka *et al.*, 2002) or show no difference (Ackermann *et al*., 2013; Pingel *et al.*, 2012) in tendinopathy compared to

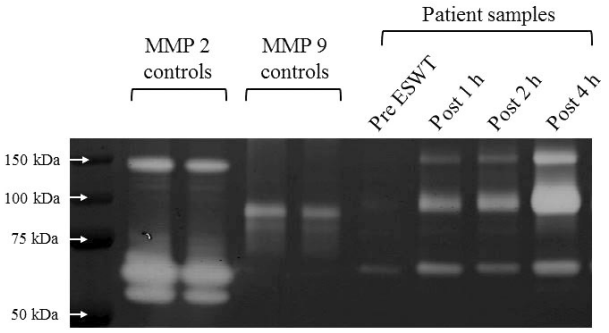

Fig. 4. Example zymogram. The protein size ladder is situated in the leftmost column followed by the results of the MMP standards in columns 2-5 and microdialysis samples in columns 6-9.

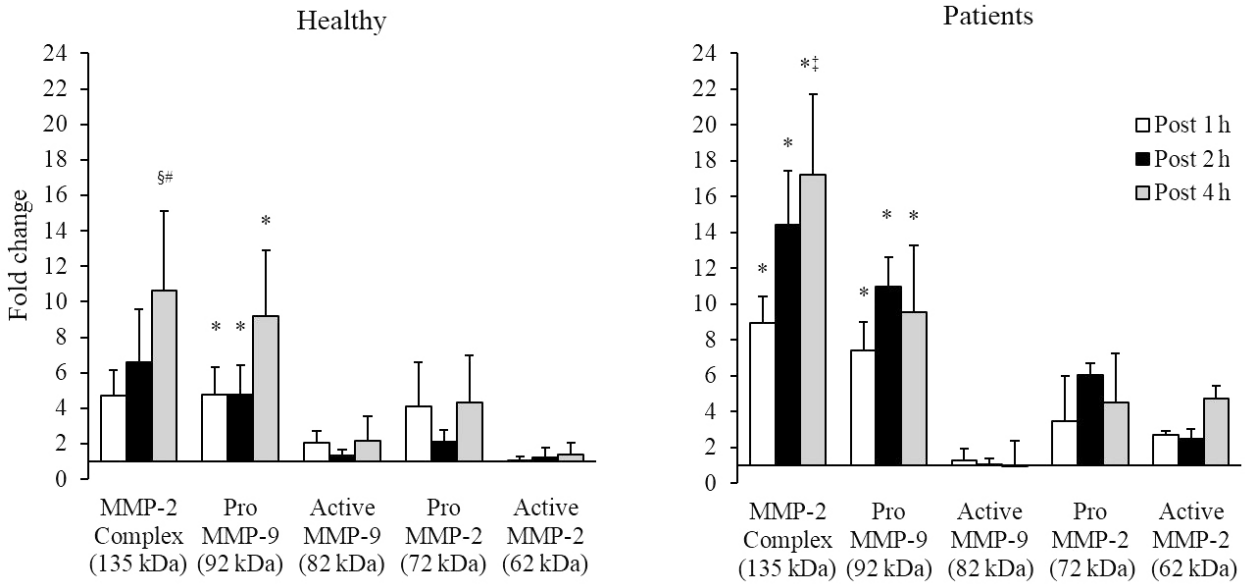

Fig. 5. Fold change in matrix metalloprotease (MMP) expression before and after a single session of ESWT in healthy ($n = 18$) and patient ($n = 10$) tendons. Expression relative to a baseline expression of 1. * Different from baseline value ($p < 0.05$); § different from post 1 h ($p < 0.05$); ‡ different from post 1 h ($p < 0.1$); # different from post 2 h ($p < 0.05$).

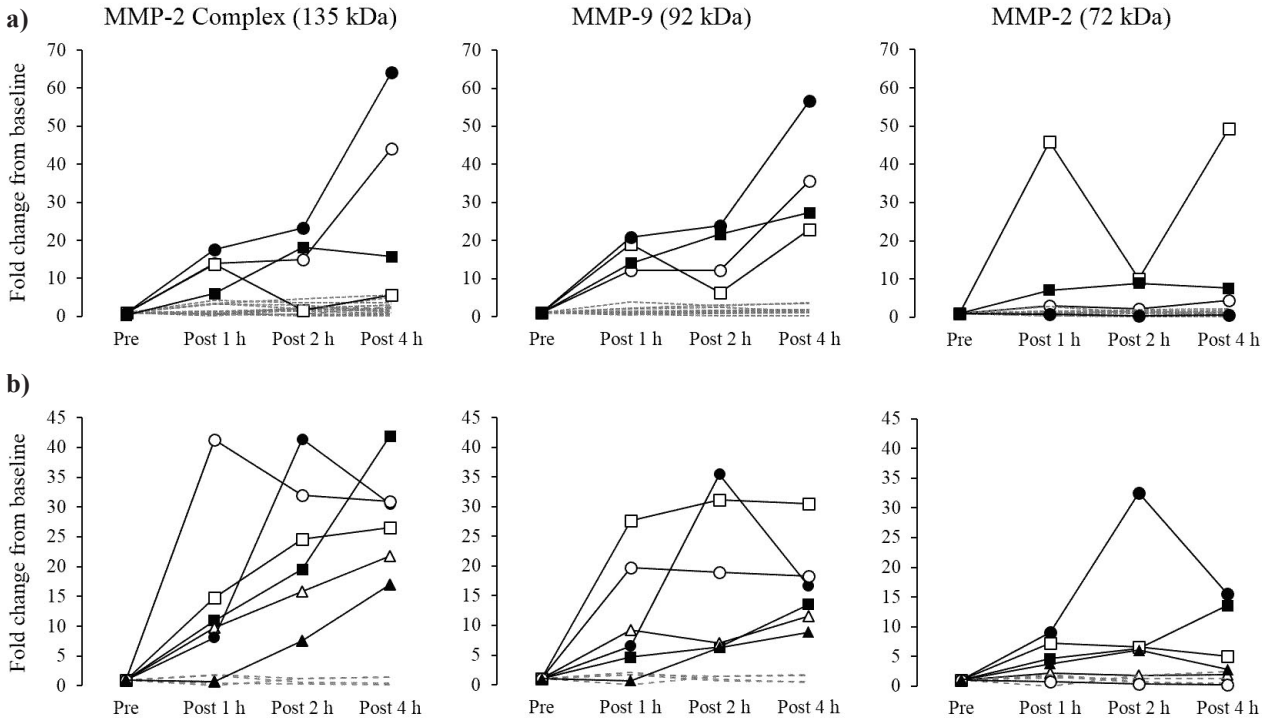

Fig. 6. Change in MMP expression from baseline (pre-ESWT) in **a**) healthy (n = 18) and **b**) patient (n = 10) tendons, represented as responders (solid black lines) and non-responders (dashed grey lines). Responders were classified as demonstrating a minimum 5-fold increase in any MMP at any time point post-ESWT when compared to baseline values. Individual responders are presented with a unique marker, traceable across graphs. Approximately 4/18 (22 %) and 6/10 (60 %) of healthy and patient tendons, respectively, may be considered 'responders' to ESWT.

healthy tendons. Similarly, IL-6 may be greater (Legerlotz *et al.*, 2012; Millar *et al*., 2009) or no different (Pingel *et al*., 2012) in tendinopathy.

Concentrations of IL-6 and IL-8 increased significantly after ESWT and showed no sign of returning to baseline within the post-ESWT sampling period. There was no significant difference in the cytokine response between healthy and patient tendons. Our findings are in contrast to those of Han *et al*. (2009), who reported a decrease in IL-6 after ESWT was administered to tenocytes cultured from tendinopathic human Achilles tendons, but no change in IL-6 in cells cultured from healthy tendon. In the same study, IL-1β increased ~4-fold in tenocytes from healthy Achilles after ESWT, which is also in contrast to our finding that IL-1β did not change in either group at any time point examined. These disagreements between studies may be due to differences in the *in vitro* and *in vivo* experiments. Firstly, the cultured tenocytes were not in a physiologically or mechanically relevant environment, therefore unlikely to respond in the same way as they would *in situ*. Moreover, the authors do not describe which of the four shockwave conditions (250, 500, 1000 or 2000 shocks) their IL-6 data represents, so it is difficult to compare this data to our results and the findings of other studies. Lastly, different devices were used to generate the shockwaves in each study, which have been shown to produce pressure waves with distinctly different physical characteristics (van der Worp *et al.*, 2013). Specifically, the energy flux density of pressure waves delivered using a radial handpiece is highest at the applicator tip and reduces the further it travels due to a convex waveform (current study), whereas pressure waves delivered using a focused handpiece converge to concentrate energy at a specific depth (Schmitz *et al*., 2013). Currently, there is a lack of research detailing the significance of pressure wave shape and delivery method on tendon tissue biology (Maier and Schmitz, 2008; van der Worp *et al*., 2013), although the response of tenocyte monolayers to soft-focused shockwaves (a third type of shockwave which maintains the temporal characteristics of a focused shockwave, but applies the stimulus to a larger focal area) was recently examined (de Girolamo *et al.*, 2014). Not only did this study deliver the same total energy as the current study, but the monolayer provided some cell-to-cell contact which is important for mechanotransduction. The authors reported an increase in IL-6 24 h after shockwave administration, which is in support of our findings.

IL-6 is a multifunctional inflammatory cytokine, which demonstrates both pro- and anti-inflammatory actions and is released in response to mechanical loading. *In vitro*, human tenocytes demonstrate increased levels of IL-6 in response to cyclic stretching (Legerlotz *et al.*, 2013; Skutek *et al.*, 2001). Further, exercise-induced increases in IL-6 were found *in vivo* in the Achilles peritendinous space after a period of running (Langberg *et al*., 2002b). Importantly, IL-6 has been shown to stimulate fibroblasts to increase the production of various extracellular matrix (ECM) components (Duncan and Berman, 1991) including

collagen, with and without the presence of a mechanical stimulus (Andersen *et al*., 2011), suggesting that IL-6 is a key regulator of connective tissue health. Given the mechanical nature of shockwaves, an increase in IL-6 after ESWT may facilitate tendon adaptation (Andersen *et al*., 2011; Lin *et al.*, 2005) or healing processes (Ackermann *et al.*, 2013; Lin *et al.*, 2006). Whilst there are many studies advocating the role of IL-6 in tendon adaptation, IL-6 may also promote negative effects. IL-6 has been shown to play a role in fibroblast proliferation (Mihara *et al.*, 1995) and neoangiogenesis during tendon healing (Nakama *et al.*, 2006), which have been implicated in tendinopathy. These conflicting arguments demonstrate the necessity for further research into tendinopathy-specific inflammatory and healing pathways before extensive conclusions can be drawn from our data.

To our knowledge, this is the first study to report an IL-8 response to ESWT in any connective tissue. IL-8 is an important pro-inflammatory chemokine, which can be stored and released from many cell types, including neutrophils and activated fibroblasts (Hoffmann *et al.*, 2002). It has a potent chemoattractant activity, which attracts and activates neutrophils, triggering further IL-8 release (Masure *et al.*, 1991). The rapid infiltration of neutrophils to a tissue is considered the primary inflammatory response and neutrophil degranulation releases enzymes which begin the process of degrading injured tissue. Although IL-8 can be rapidly induced by pathogens (Eckmann *et al.*, 1993) and pro-inflammatory cytokines TNF-α and IL-1β (Kasahara *et al.*, 1991), we suspect from our results that the mechanism is mechanical in nature. There is a surprising lack of literature to support the induction of IL-8 by mechanical loading in connective tissue, given that the biological mechanisms of IL-8 are well documented (Baggiolini and Clark-Lewis, 1992). However, mechanically induced up-regulation of IL-8 is not new; shear stresses and mechanical stretch increase IL-8 gene expression (Cheng *et al.*, 2007) and secretion (Iwaki *et al.*, 2009; Okada *et al.*, 1998) in vascular endothelial cells, while vibration and oscillatory pressure changes increase IL-8 secretion in bronchial epithelium (Huang *et al.*, 2012; Puig *et al.*, 2005).

An additional feature of shockwave therapy that has not yet been discussed in relation to these study results is the deformation to the tendon resulting from pressure with which the applicator tip is held against the tendon. As tenocytes perceive tissue deformation resulting from mechanical loading rather than the actual force applied, and given the particularly superficial nature of the tendons investigated here, the potential for this effect may be more pertinent in the present study. Unfortunately, this application pressure was not controlled for presently, and the stiffness of the tissues was not known, therefore any resulting tissue deformation could not be estimated. Nonetheless, the potential for this effect should be considered in future biological studies.

Matrix metalloproteinase activity

MMPs are involved in tendon tissue turnover and, in balance with their tissue inhibitors (TIMPs), are important in maintaining tendon homeostasis. MMP-2 activity was detected at ~135, 72 and 62 kDa and MMP-9 activity at 92 and 82 kDa, confirmed with EDTA controls. Consistent with previous studies, pro-MMP forms were more strongly detected than active forms (Koskinen *et al*., 2004). This is likely due to the fact that: 1) only basal levels of active MMPs are required to perform the proteolytic activities required for constant ECM remodelling (Bode and Maskos, 2003), unless the tissue is responding to injury or changes in loading (Snoek-van Beurden and Von den Hoff, 2005); and 2) MMPs are synthesised in a latent form, and once secreted, require extracellular activation by other MMPs.

Lower resting MMP-9 (82 kDa) activity was found in tendinopathy patients in the current study whilst no difference was found for MMP-2. Whilst overproduction of MMPs may lead to tissue destruction in chronic inflammatory conditions (*e.g.* osteoarthritis; Tetlow *et al.*, 2001), underproduction may suggest an accumulation of pathological tissue. This hypothesis is in keeping with the histopathological findings of degenerative tendinopathy, which include collagen disorganisation and an increase in ECM constituents (Järvinen *et al.*, 1997; Khan *et al.*, 1999). In support of our findings, Riley *et al.* (2002) reported lower MMP-9 activity in pathological human tendons, whilst Jones *et al.* (2006) found no difference in MMP-2 mRNA expression in normal, degenerate and ruptured human tendons. However, these findings are somewhat in contrast to other studies. Pingel *et al*. (2012) reported significantly elevated MMP-2 and MMP-9 mRNA expression in tendinopathic biopsies compared to healthy biopsies from human Achilles tendons. Parkinson *et al.* (2010) found an increased MMP-9 mRNA expression in chronic patellar tendinopathy. Jones *et al*. (2006) described higher levels of MMP-9 mRNA in ruptured compared with normal tendons, but no difference in tendinopathy. However, MMP activity is controlled on many levels and an increase in mRNA expression does not guarantee an increase in protein synthesis (Snoek-van Beurden and Von den Hoff, 2005), therefore we are limited in our ability to compare studies.

An increase in the MMP-2 component with the largest molecular mass (~135 kDa) was found relative to baseline after ESWT in both healthy and patient groups. MMP-2 has been shown to form a reduction-sensitive homodimer, whose high molecular mass is approximately twice that of the active monomer (Koo *et al.*, 2012). Dimerisation has been suggested as a mechanism for regulating MMP-2 activity and is thought to occur intracellularly, thus the homodimer is secreted into the ECM as an inactive MMP-2 form (Koo *et al.,* 2012). Interestingly, neither pro- nor active MMP-2 increased significantly with ESWT, although increases in MMP-2 may not occur as rapidly as other MMPs; up-regulation of both pro- and active MMP-2 was only seen after two weeks of running in the rat supraspinatus (Attia *et al.*, 2013) or three days of running intervention in the human Achilles tendon (Koskinen *et al.,* 2004). Alternatively, microdialysis may not be an optimal technique for detecting MMP-2 activity due to the cell-bound mechanisms by which it is recruited and activated (Nagase, 1998).

In contrast to the MMP-2 monomers, a marked increase in pro-MMP-9 expression was found immediately after

ESWT. Similar findings have been reported after a period of running exercise (Koskinen *et al.,* 2004). In the present study, such large increases in pro-MMP-9 over the short time-frame from which dialysate was acquired suggests that intracellularly-stored MMP-9 is secreted into the extracellular space. However, levels of active MMP-9 did not change after ESWT, suggesting that latent forms are not automatically immediately activated. It is generally accepted that MMP-9 is produced and stored in its pro-form in the tertiary granules of neutrophils (Masure *et al.,* 1991) and is rapidly released after IL-8-mediated neutrophil activation (Hasty *et al.*, 1990). The increase in pro-MMP 9 in the current study may be a result of the increase in IL-8 seen immediately after ESWT administration, although TGF-β1 – an important mediator in mechanotransduction (Heinemeier *et al.*, 2003) – has also been implicated in the mechanical regulation of MMPs (Jones *et al.,* 2013). In keeping with the hypothesis that basal reparative activity is reduced in degenerative tendinopathy, an increase in MMP activity from baseline might indicate a positive mechanism, increasing the likelihood of tissue regeneration by increasing the pro-MMP forms availability for activation. However, TIMP activity was not examined currently, therefore we are limited in our ability to draw conclusions regarding these changes.

Responders *versus* non-responders

The lack of significance associated with the change in MMP activity in the current study might appear surprising given the clear increased activity observed after ESWT in some individuals. However, it is evident there are large inter-individual differences in the MMP response due to possible responders and non-responders (Fig. 6). In the case of our results, responders were identified as individuals demonstrating a minimum 5-fold increase in any MMP at any time point post-ESWT when compared to baseline values. Although we recognise the limitations and assumptions associated with categorising individual tendon responses from our limited data set in this manner, this arbitrary threshold was chosen based on a natural separation of data around this point, leading to an intuitive method of characterising a complex dataset. The responder phenomenon becomes particularly interesting when considering the clinical effectiveness of ESWT. Based on good or excellent improvement in condition symptoms following a ESWT treatment course, success rates of 52-85 % for non-insertional Achilles (Furia, 2008; Rompe *et al.*, 2007; Saxena *et al.*, 2011; Vulpiani *et al.*, 2009) and 50-74 % for patellar tendinopathies (Peers *et al.*, 2003; Vulpiani *et al.*, 2007; Wang *et al*., 2007) are reported, which are similar to the percentage of patient tendons that responded to ESWT in the present study (60 %). It is possible that individuals whose tendinopathies improved with ESWT are individuals who would demonstrate a biological response to treatment. However, it should be emphasised that the response observed presently is to a single session of ESWT and may not necessarily reflect the individual's response to a typical course of treatment. Unfortunately, we do not have the data necessary to investigate the relationship between molecular response and treatment effectiveness further as this was a somewhat surprising finding. Nonetheless, as this information could help optimise treatment results by informing dosage decisions, the relationship between treatment response and treatment outcome is of potential importance and should be made a priority for future research.

Limitations

Microdialysis allows continuous *in vivo* measurements of soluble molecules in the interstitial tissue space and therefore the time course of a tissue response to stimuli to be determined. However, there are limitations to its use as a sampling tool. Although minimally invasive, there is likely to be some local tissue trauma in response to positioning the microdialysis probe. Transient increases in inflammatory mediators TGF-1β (Heinemeier *et al.,* 2003), PGE_2 and TXB_2 (Langberg *et al.,* 1999) have been identified immediately after catheter insertion, with mediator concentrations returning to baseline levels after ~120-150 min. The effect of local tissue trauma was not examined for the interleukins or MMPs investigated in the present study, although we have carried out an additional study in which we confirmed that MMP activity is unchanged in the 90 min after probe insertion (Fullerton *et al.*, 2014). In addition, samples used for investigating the interleukin baseline were not collected until 90-120 min post probe insertion, which should minimise the inflammatory response to catheter insertion based on previously published work and study protocols.

In addition to this limitation, whilst microdialysis aims to characterise the local intercellular water space based on the movement of unbound water-soluble substances across a semipermeable membrane, this space is largely continuous and thus we are unable to categorically state that the origin of the molecules present in the dialysate was from the tendon itself. Whilst we cannot differentiate between molecules that have diffused from the tendon and those from other local tissues, it has been shown previously that the tendon periphery is more metabolically active than the tendon core (Langberg *et al.*, 2002a), thus positioning the microdialysis probe in the peritendinous space is likely to be more representative of tendon biology than any other location.

Lastly, we did not screen individuals for asymptomatic tendinopathies using imaging modalities. Therefore, it is possible that individuals with non-symptomatic tendinopathies are included in our healthy tendon group. However, the prevalence of asymptomatic tendinopathy is relatively low in active young adults (3.8 %, Joseph *et al.*, 2012) and the ability of imaging techniques to predict future symptomatic tendinopathy has also been questioned (Comin *et al.*, 2013).

Conclusion

To our knowledge, these findings provide a novel insight into the biological mechanisms underpinning the observed clinical effects of ESWT in humans *in vivo*. Our findings suggest that the mechanical stimulus provided by ESWT might aid the initiation of tendon regeneration in tendinopathy by promoting pro-inflammatory and

catabolic processes that are associated with removing damaged matrix constituents. Our findings are supported by the association between IL-6 and collagen synthesis, and the IL-8 cascade which encourages neutrophils to release ECM-degrading enzymes. The response profile and time course of other biological indicators of matrix turnover and tendon regeneration are required to build upon the present findings, as are the effects of different ESWT treatment dosage protocols and devices. Our results also suggest the possibility of biological responders and non-responders to the ESWT protocol used presently, but requires further investigation to substantiate this claim. Knowledge of these mechanisms has the potential to help improve clinical paradigms concerning treatment decisions and protocols and therefore optimise efficacy for patients.

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Discussion with Reviewers

Reviewer II: The manuscript confirms the results of previous *in vitro* studies and brings new knowledge on the *in vivo* effect of ESWT in healthy and pathologic tendons. The expression of different cytokines induced by shock waves, in this as in other studies, seems to confirm that an adequate stimulus modulates the "physiological inflammatory sequence" which characterises the tendon healing process. Nevertheless, as described, the role of microdialysis seems to be restrictive to the earliest phases with respect to the molecular kinetics of the whole process. Finally, the manuscript highlights the question of the biological responders and non-responders to ESWT. This would give new impulse to the debate on the causes of the individual answers (success or failure) to physical stimuli in the treatment of tendinopathies.

Authors: We thank the reviewer for these comments and appreciate that they recognise the potential importance of our findings, in respect to both verifying the presence of cytokines in initiating tendon healing, and in evidencing that individuals may respond individually to treatment modalities. Whilst we agree that the role of microdialysis in determining some of the biological events that may underpin ESWT is limited, we hope that our study stimulates further research in the area and has identified avenues that should take priority for future research.

Reviewer III: Given the low number of participants, is it relevant to divide into non-responders and responders?

Authors: Although the patient group is not ideal with respect to size and homogeneity, we think that our findings are interesting enough to report. Moreover, the potential importance of these surprising findings may provide a rationale for future research.

Reviewer III: Is shock wave therapy effective in tendinopathy conditions other than those that have calcifications? Does it make sense to study biological changes in tendinopathy when we do not know whether the treatment is effective?

Authors: Shockwave therapy has been proven to be an effective treatment for non-calcific tendinopathy The results from our recent systematic review regarding this matter suggests that there is enough evidence from high-quality studies evaluating the effectiveness of ESWT for lower limb tendinopathies (Achilles, patellar and greater trochanter) to conclude that ESWT is an effective intervention for tendinopathies (Mani-Babu *et al*., 2014). We conclude, based on the findings from this review, that the biological underpinning of ESWT continue to be investigated.

Reviewer III: Does peritendinous microdialysis reflect what goes on inside the tendon or does it rather reflect what happens along the tendon in for example fat tissue?

Authors: Microdialysis enables an analysis of local tissue biology *in vivo* whilst providing a new avenue to explore biological events in real time. It has been shown previously that the tendon periphery is more metabolically active than the tendon core (Langberg *et al*., 2002a; Rempel and Abrahamsson, 2001), and so peritendinous microdialysis is likely to be more representative of tendon biology from this perspective. However, we cannot differentiate between molecules that have diffused from the tendon and those from other local tissues such as fat, which is a methodological limitation.

Additional Reference

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