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REGULAR ARTICLE

Extracorporeal shockwave-induced expression of lubricin in tendons and septa

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Abstract Lubricin, a lubricating glycoprotein that facilitates tendon gliding, is upregulated by mechanical as well as biochemical stimuli, prompting this study of its induction by extracorporeal shockwave therapy (ESWT). The objective of this study was to characterize and quantify the effect of ESWT on lubricin expression in tendons and septa in a rat model. Hindlimbs of six rats were treated with low-dose ESWT and those of another six with high-dose ESWT, using contralateral limbs as controls. After 4 days, resected samples were processed for immunolocalization of lubricin using a purified monoclonal antibody. ESWT was found to increase lubricin expression in both low-dose and high-dose ESWT-treated tendons and also in septa. Lubricin expression generally increased with increasing dose of ESWT. Increased lubricin expression may contribute to the beneficial effects of ESWT in providing pain and symptom relief in musculoskeletal disorders by decreasing erosive wear.

Keywords ESWT · Lubricin · Tendon · Septum · Rat

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Introduction

Lubricin, a lubricating molecule in diarthrodial joints, has been shown to enhance tendon-gliding in vitro (Zhao et al. 2010) and in vivo (Kohrs et al. 2011). Lubricin is a mucinous glycoprotein initially found to be produced by synovial cells (Rhee et al. 2005) and isolated from synovial fluid (Swann et al. 1985). Superficial zone protein (SZP), produced by superficial zone chondrocytes (Schumacher et al. 1994), is homologous to lubricin (Jay et al. 2001) and megakaryocyte-stimulating factor (MSF) (Flannery et al. 1999), all of which are products of the megakaryocyte-stimulating factor gene (*msf*) (Jay et al. 2001). Collectively, these homologous glycoproteins have been referred to as proteoglycan 4 and are encoded by 12 exons of the gene *msf*, also known as *Prg4* (Englert et al. 2005). Lubricin, which has been found to demonstrate anti-adhesion (Englert et al. 2005) as well as lubricating properties, has been reported in bovine ligaments (Lee et al. 2008a) and in canine tendons (Sun et al. 2006). Its presence within fascicles and fascicular sheaths in caprine (Funakoshi et al. 2008) and human (Funakoshi and Spector 2010) tendons suggested a role in interfascicular tribology.

Certain mechanical (Sun et al. 2006) and biochemical stimuli (Jones and Flannery 2007; Lee et al. 2008b) have been shown to regulate lubricin expression in vivo, prompting this investigation of the effects of extracorporeal shockwave therapy (ESWT) on lubricin expression in tendons. ESWs are pressure waves of short duration in

which a high-amplitude compression peak is immediately followed by a lower-magnitude tensile wave (Ogden et al. 2001a). ESWT has proven effective for the treatment of a number of musculoskeletal disorders, including plantar fasciitis (Ogden et al. 2001b), calcific tendonitis of the rotator cuff (Rompe et al. 1998), Achilles tendonitis (Hart 2009) and nonunion of long bone fractures (Schaden et al. 2001). Despite its efficacy, the molecular and cellular mechanisms by which ESWT achieves its beneficial therapeutic effects remain poorly understood. Current proposed mechanisms for the role of ESWs in tissue healing and regeneration involve tenocyte proliferation and collagen metabolism (Chen et al. 2004), increased collagen turnover and accelerated tissue healing (Bosch et al. 2007) and enhanced neovascularization (Wang et al. 2003a). That ESWT has been found to upregulate select cytokines including TGF-β1 (Chen et al. 2004), which is known to stimulate the expression of lubricin (Lee et al. 2008a; Jones and Flannery 2007) and promotes the healing in conditions including tendinitis (Chen et al. 2004; Chen et al. 2003; Wang et al. 2003b), supports the supposition that ESWT may induce lubricin expression in tendons.

The rat hindlimb model was chosen in order to evaluate lubricin in several different types of tendons in the same sample. The septa within the same hindlimbs were also evaluated as comparative controls, because they also comprise bands of fibrocollagenous tissue. It may be the case that no single mechanism fully explains the underlying biological processes; the beneficial effects of ESWT may result from the additive effects of a number of biological mechanisms. ESWT-induced expression of lubricin in tendons and septa may be an alternative mechanism of symptomatic relief through increasing lubrication, both among adjacent musculoskeletal structures and among collagen bundles within structures and decreasing erosive wear in tissues.

The objective of this study was to test the hypothesis that lubricin expression increases following ESWT in tendons and septa and to answer the following related questions: (1) is the effect of ESWT on lubricin expression dose-dependent? (2) does the effect of ESWT on lubricin expression vary among the tendons and septa? and (3) can lubricin be found intracellularly in the tendons and septa, suggesting endogenous production in these tissues?

Materials & methods

Study design

All experiments were conducted with IACUC approval. Twelve Sprague–Dawley male rats, aged 4 months and weighing 400–450 g, divided into two experimental groups based on ESW dose, were used in this study. Focused ESWs were generated using a high-energy (0.4 mJ/mm^2) electrohydraulic shockwave source (OssaTron; SANUWAVE, Alpharetta, GA, USA) for high-dose and a low-energy (0.15 mJ/mm^2) electrohydraulic shockwave source (EvoTron; SANUWAVE) for low-dose ESWs. The EvoTron was first employed for low-dose ESWs but subsequent high-dose experiments necessitated the Ossatron, thus the use of two different devices. The ellipsoid shaped focal zone was approximately 1 cm in diameter and 5 cm long. The methodology used in these studies is based on a similar approach used by Chen et al. to stimulate tenocyte cell proliferation in rats (Chen et al. 2004). Animals were treated in one session using 3,000 shocks at the respective energy density, at a firing rate of 4 s^{-1}. Sections of the hindlimb within the ESW focus were taken for histological analysis and evaluated to yield n=12 for the controls (i.e., non-ESW-treated limbs) and n=6 for the low- and high-dose samples. Problems associated with specimen processing resulted in the loss of certain tendons and septa.

Animal model and experimental procedures

Rats were anesthetized using 2% isoflurane and placed in the prone position for shockwave treatment. The rat's left hindlimb was shaved prior to shockwave application and ultrasound acoustic coupling gel was applied to the skin at the treatment site. The shockwave device was positioned on the posterolateral side of the hindlimb at 1.5 cm distal to the center of the knee joint. The coupling pressure in both devices was set so that the secondary [treatment] focus was at the membrane front, which was coupled to the skin with coupling gel. Coupling in a water bath was not employed, for the sake of clinical relevance. For all animals, the contralateral limb served as control. All rats were euthanized by CO_2 4 days post-treatment, a time point chosen based on previous data on ESWT induced new periosteal bone formation in rats (Takahashi et al. 2004; Kearney et al. 2011).

Immunohistochemistry preparation and staining

The excised limbs were fixed in 10% buffered formalin and decalcified using 10% formic acid. Samples of normal rat articular cartilage were similarly processed to serve as controls for lubricin staining. The samples were embedded in paraffin and sections were cut in the transverse plane across the hindlimbs to a 7-μm thickness with a microtome (Shandon Finesse Model ME+; Thermo Fisher Scientific, Waltham, MA, USA), deparaffinized in xylene and rehydrated in reagent alcohol. Hematoxylin and eosin staining was performed for cell morphology and identification and Masson's trichrome staining was used to reveal collagen and to demonstrate the anatomy of the rat hindlimb (Fig. 1a) with the locations of the various tendons and

septa in the transverse sections through the rat hindlimb (Fig. 1b and c).

Immunohistochemistry was performed with the following autostaining process, punctuated by rinses with Tris-buffered saline solution containing Tween (TBS, S3006; Dako, Carpinteria, CA). Samples were treated with 0.1% protease (type XIV, P5147; Sigma, St. Louis, Missouri) for 40 minutes, followed by 3% hydrogen peroxide for 10 minutes and protein block serum-free (X0909; Dako). The slides were then incubated with a primary anti-lubricin monoclonal antibody (S6.79, provided by the Rush University Medical Center, Chicago, IL, USA) or with a negative mouse immunoglobulin-2b (IgG2b) control (X0944; Dako) for 15 minutes. The S6.79 anti-lubricin antibody is an IgG2b immunoglobulin developed in the mouse against human lubricin, found to react with a variety of mammalian lubricin molecules, including human, dog, bovine, guinea pig and rabbit (Su et al. 2001). The sections were then incubated with a biotinylated secondary antibody for 15 minutes, followed by streptavidin-conjugated horseradish peroxidase for 15 minutes. Labeling was achieved using an aminoethyl carbazole (AEC) chromogen kit (K3461; Dako). AEC was applied to the sections for 10 minutes to allow detection of lubricin by the presence of red chromogen at the antigen sites in the tissue. Counterstaining was performed with Mayer's hematoxylin and coverslips were applied with an aqueous mounting medium (Faramount; Dako).

Histological evaluation

Light microscopy was used to visualize the tissues. Digital micrographs were acquired using a MicroFire camera (model S99809; Meyer Instruments, Houston, TX, USA) mounted on an Olympus microscope (BX51; Olympus, Tokyo, Japan). Tendons and septa were identified upon light microscopy by histological morphology and anatomic location. Lubricin expression in tendons and septa was evaluated separately in the extracellular matrix (ECM) and intracellularly (IC). IC-positive staining was defined as the presence of red chromogen at the border of the hematoxylin-stained nucleus or distributed within the cytoplasm. To evaluate the areal percentage of the ECM staining for lubricin and the percentage of cells displaying IC staining for lubricin, samples were graded semiquantitatively on a scale from 0–5, where 0 denotes the absence of lubricin staining in the tissue area or the absence of lubricin-containing cells, 1 denotes trace lubricin-positive staining involving <5% of the tissue area or <5% of the cells, 2 denotes lubricin-positive staining in 5–25% of the total tissue area or percentage of cells, 3 denotes that 26–50% stained positively, 4 denotes that 51–75% stained positively and 5 denotes that 76–100% stained positively. Each tendon and septum included in the study had a minimum of 50 cells. All measurements were recorded by one evaluator (DZ), with selected concurrences by two subsequent observers (TC, MS). Since no assessment of interobserver variability was made for any of the measurements, we did not base the differences among the groups on fine discriminations of the data. The following tendons and septa were evaluated:

Tendons: gracilis (g), semitendinosis (st), flexor digitorum longus (fdl), tibialis cranialis (tcn), tibialis caudalis (tcd), extensor digitorum longus (edl), peroneus longus (pl), biceps femoris (bf), soleus (s). The side of the pl tendon closer to the anterior intermuscular septum (pl-s) was evaluated separately from the side closer to the fibula (pl-f).

Septa: interosseous membrane (iom), anterior intermuscular septum (ais), transverse intermuscular septum (tis). The tibial side of the iom (iom-t) was evaluated separately from the fibular side (iom-f).

Statistical methods

We proposed to use six animals per group. Our power calculation for sample size determination was based on the desire to determine a significant as 30% difference in a selected outcome variable assuming a 15% standard deviation, with $\alpha=0.05$ and $\beta=0.05$. Our supposition was that a difference between groups of more than 30% for a particular outcome variable would be necessary for ESW to be of posible clinical importance.

The Wilcoxon signed-rank test was employed for the paired data comparing the ESW dose and control in the same animals.

Results

In support of our hypothesis, positive immunostaining for lubricin (Fig. 2) was observed in all of the tendons and septa of the low-dose (Fig. 3b) and high-dose (Fig. 3c) ESWT hindlimbs, compared to the majority of the non-ESW-treated control tendon and septa samples that were negative for lubricin. When positive staining was observed in control tissues, it was present mostly in trace amounts (Fig. 2). The mean ECM and IC lubricin values were calculated for the tendons and septa for the three dose groups (Table 1). As these were non-parametric data, the mean values were employed for summary assessments only, not for statistical analyses. The mean values for the ESW-treated tendons and septa were notably higher than for the non-treated controls. Wilcoxon signed-rank testing revealed a significant effect of ESWT on lubricin expression in the ECM and IC in tendons (both p-values <0.0001) and septa (ECM: $p=0.001$, IC: $p=$

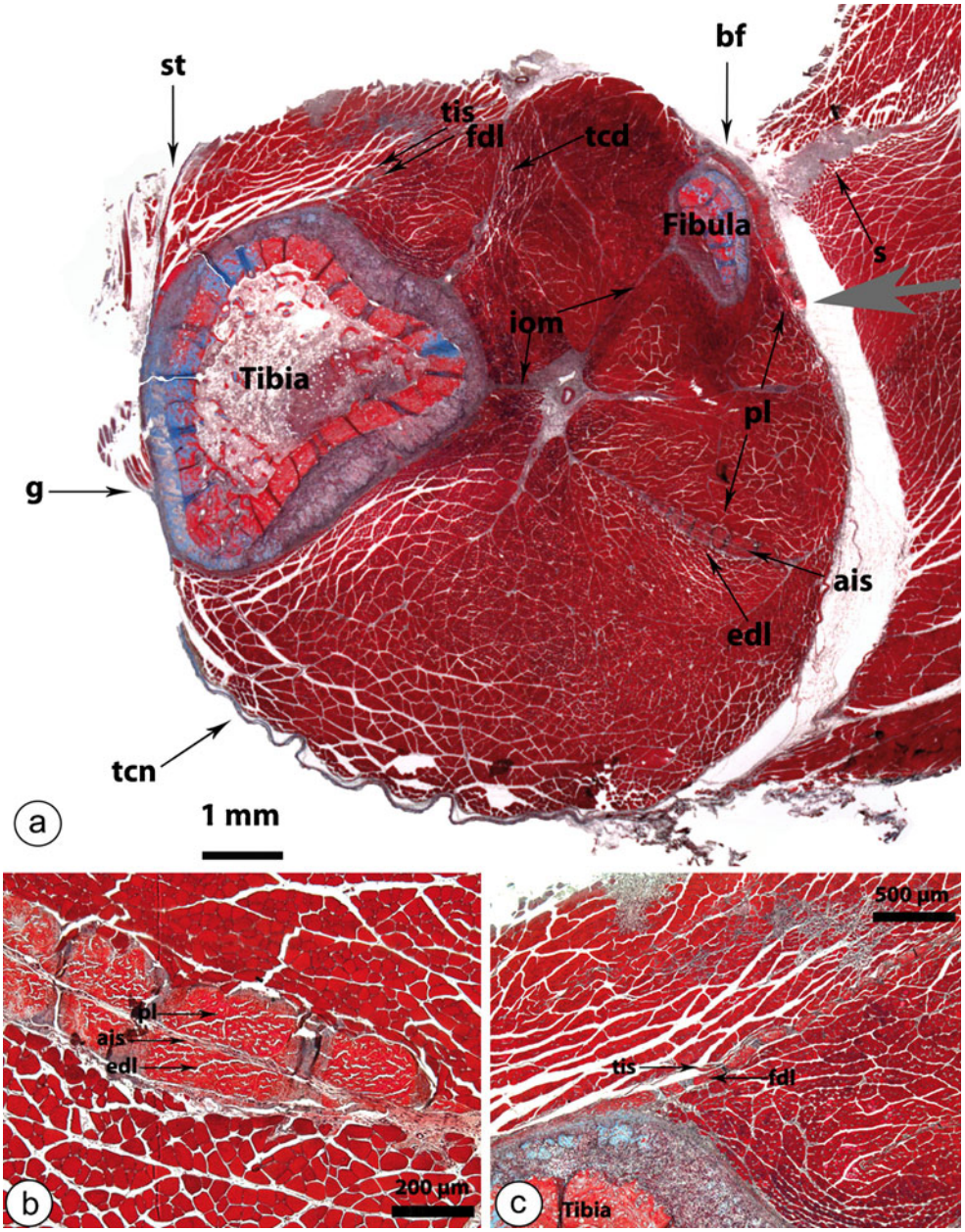

Fig. 1 **a** The low-magnification micrograph of the rat hindlimb shows notable anatomic structures. The *gray arrow* indicates the direction of applied ESWs. **b**,**c** The high-magnification micrograph of tendons and septum highlights histological differences (Masson's trichome stain). 12.5×, 40×, 12.5× magnification

0.003). None of the immunohistochemical negative control sections showed the red chromogen. Lubricin was consistently seen as a discrete layer on rat articular cartilage positive control samples in accordance with a previous study (Zhang et al. 2007) (data not shown). It is notable that fresh cut surfaces of the tissues, produced during trimming of the samples in preparation for paraffin embedment, did not stain for lubricin, indicating that the presence of lubricin on tissue borders was not the result of edge-artifact staining.

The amount of lubricin in the ECM and the percentage of cells with IC lubricin varied among the tendons (Fig. 2a and b) and septa (Fig. 2c and d). In most tendons and septa, there was a clear increase in lubricin expression with increasing ESWT dose (Fig. 2). Intracellular lubricin staining was seen in the cytoplasm of elongated cells resembling fibroblasts typically seen in tendons and septa (Fig. 3). In all samples, the intensity of red chromogen was highest at the bony interface and the intensity of staining decreased as the distance from the bone–tendon or bone–septum interface increased.

Although there were no qualitative differences in the cell number among the experimental groups, we leave it to future studies to ascertain the effects of ESWT on cellularity.

Discussion

The principal finding of this study was that ESWT increased the amount of lubricin detected in tendons of

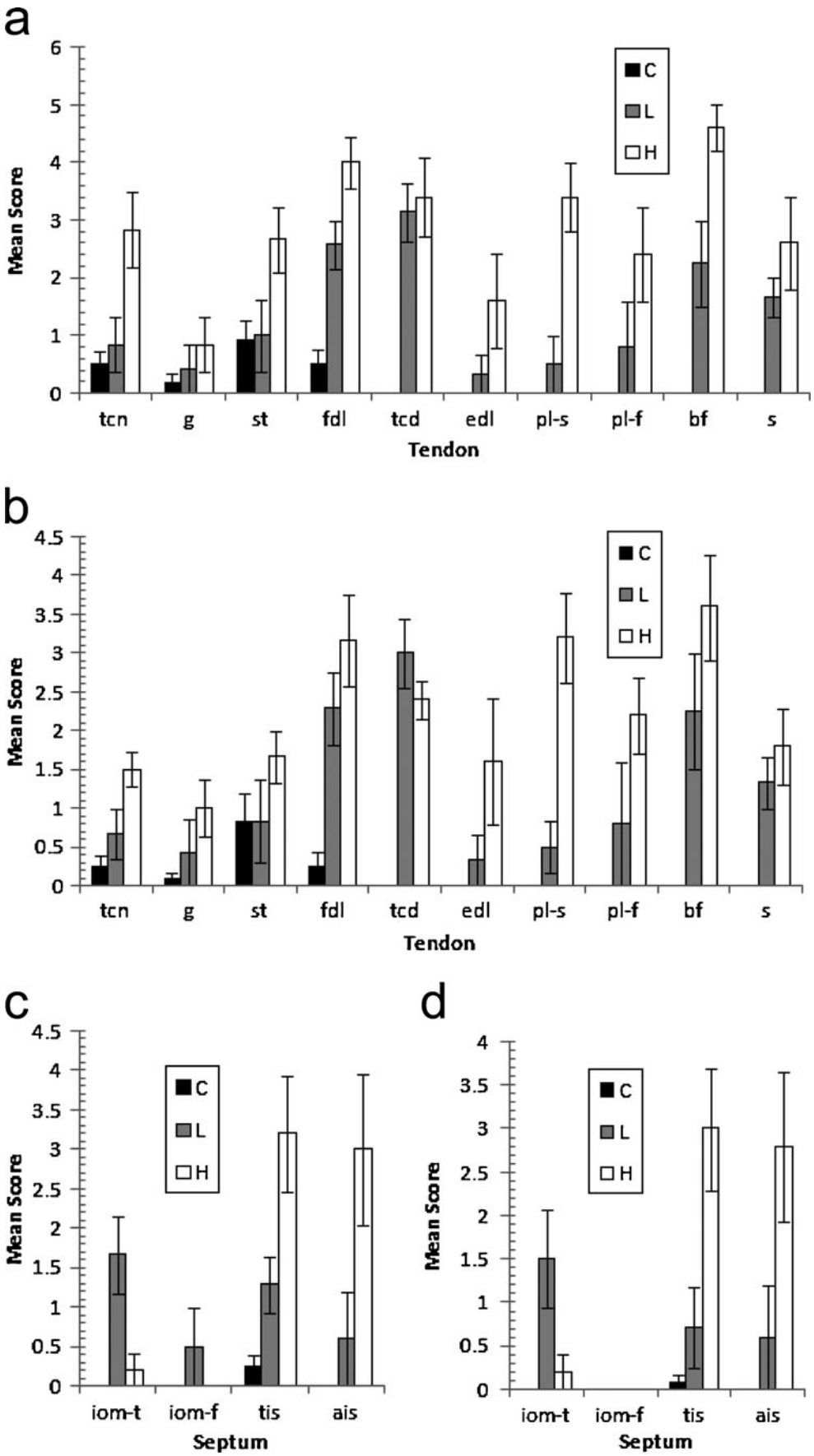

Fig. 2 The graphs show mean scores with standard error bars for lubricin expression in tendons and septa in response to shockwave therapy. **a** Tendons, ECM. **b** Tendons, IC. **c** Septa, ECM. **d** Septa, IC. Legend: *H* = high-dose ESW, *L* = low-dose ESW, *C* = control tissues

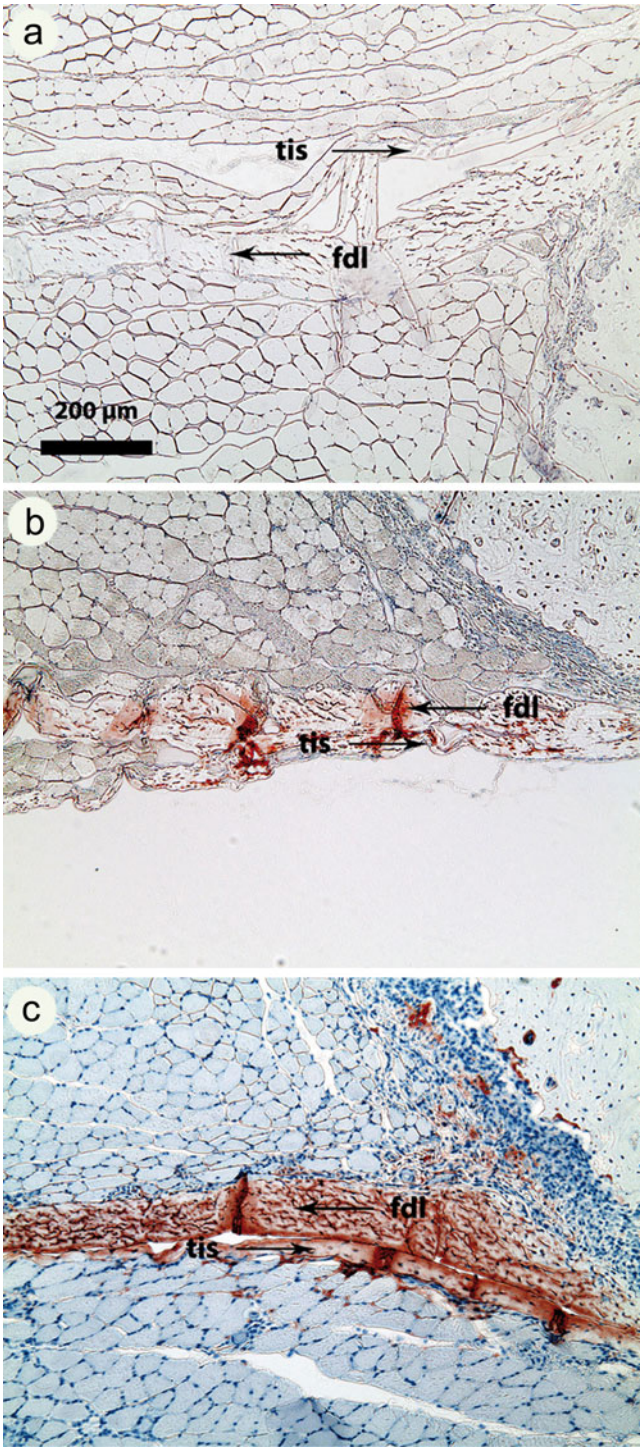

Fig. 3 The micrographs show immunostaining for lubricin in (**a**) control tissues, (**b**) low-dose and (**c**) high-dose ESW-treated tendons and septa, showing both ECM and IC staining. 40×, 40×, 40× magnification

the rat hindlimb, supporting our central hypothesis. Of note was that ESWT also increased the expression of lubricin in septa, suggesting that these tissues of similar composition but different architecture respond similarly to lubricin

Table 1 Mean scores ± standard error for the extracellular matrix and intracellular staining of lubricin in tendons and septa

	Control	Low-dose	High-dose
Extracellular matrix			
Tendons	0.28±0.08	1.35±0.20	2.82±0.23
Septa	0.10±0.05	1.15±0.25	1.68±0.46
Intracellular			
Tendons	0.19±0.06	1.25±0.19	2.19±0.18
Septa	0.03±0.03	0.85±0.28	1.58±0.43

agonists. Future studies will be required to determine the mechanism by which ESWT achieves this effect: by acting directly as a mechanical stimulus and/or indirectly through the stimulation of lubricin regulators (viz., TGF-β) to increase lubricin expression.

In addressing our first research question, the effect of the EWST dose on lubricin expression was found to be variable for the two doses employed in this study. In most tendons and septa, lubricin expression increased with increasing ESWT dose and the mean lubricin values for the high-dose ESW exceeded the values for the low-dose treatment. Additional work will be required to further quantify the effect of the ESW dose and select shockwave parameters on lubricin expression in tendons and septa, including a wider range of ESW dose. These parameters include: the energy flux density, the magnitude and duration of the compressive and tensile peaks, the number of shocks, the firing rate, the incidence angle and the number and timing of multiple ESW treatment sessions.

In regard to our second research question, the effect of ESWT on lubricin expression varied among the tendons and septa in the rat hindlimb. Differences in the response of various tendons and septa to ESWT may be due to differences in orientation of the tissue to the shockwave propagation, vascularity and acoustic impedance of the tissue. The preferential distribution of lubricin closer to the bone–tendon and bone–septum interfaces is expected, since the greatest reflection and refraction of shockwaves occurs at the interfaces between two materials with different acoustic impedances. Our finding indicates that the effects of ESWT on the level of lubricin in tendons at other locations and on other similar tissue (viz., ligaments) may be expected to vary.

Our third research question dealing with the production of lubricin by cells in the tendons and septa was clearly answered in the affirmative. It has been shown that tenocytes are capable of expressing lubricin (Sun et al. 2006; Funakoshi et al. 2008; Funakoshi and Spector 2010), consistent with our finding of trace amounts of lubricin positive staining in control hindlimbs.

There were several limitations of this study. While the sample size was large enough to provide statistically

significant results in an adequately powered study, it would be judicious to include additional samples in the future to provide assurance of the findings. A second limitation is that the study evaluated the effects of ESWT on tendons and septa at a particular location of the rat hindlimb. It will be important to follow up these results with investigations of the effects of ESWT on tendons (and ligaments) at other locations in the rat and in other species. Third, we rely on future experiments with additional time points to further elucidate the time-dependent effects of ESWT on lubricin expression. A fourth limitation is that the phenotype of the cells containing lubricin was not determined other than by morphology and no analysis of the extracellular matrix molecules was performed to provide an insight into the lubricin-binding molecules in the ECM of these tissues. Fifth, we used a single monoclonal antibody to lubricin. Although this antibody has been used in immunohistochemical studies of a wide array of tissues with consistent findings, future work should use other lubricin antibodies for comparison. Finally, the rationale for the tribological role of lubricin in tendons and septa is predicated on a lubricating function of the lubricin found in these tissues, which needs to be supported by future research of the splice variants present.

With respect to the mechanisms that may underlie our central hypothesis, previous studies have investigated the effects of various inflammatory cytokines on lubricin expression in synoviocytes (Jones and Flannery 2007) and mesenchymal progenitors (Lee et al. 2008b). While interleukin-1 (IL-1) and tumor necrosis factor-α (TNF-α) caused decreased expression of lubricin, transforming growth factor-β (TGF-β) and bone morphogenetic protein-7 (BMP-7) significantly increased lubricin synthesis, secretion and cartilage boundary function (Jones and Flannery 2007; Lee et al. 2008b). ESWT has previously been shown to upregulate TGF-β at the protein level in tenocytes (Chen et al. 2004) and BMP-2, BMP-3, BMP-4, and BMP-7 at the mRNA and protein levels in mesenchymal cells, chondrocytes and osteoblasts (Chen et al. 2003; Wang et al. 2003b). Therefore, a possible mechanism for the upregulation of lubricin expression by ESWT involves the induction of appropriate cytokines: ESWT may increase TGF-β and BMP-7 expression in fibroblast-like cells in tendons and septa, which in turn increases lubricin expression in the local tissue. Additionally, ESWT has been hypothesized to induce an acute biochemical activation of latent matrix metalloproteinases (MMPs) (Bosch et al. 2009). MMPs are known to activate latent TGF-β sequestered in the ECM (Jenkins 2008), possibly leading to lubricin upregulation. A second mechanism for the increased lubricin expression involves direct stimulation of fibroblasts in tendons and septa from the mechanical stress of shockwave treatment and the attendant cavitation process. Previous studies have shown that mechanical stimuli regulate lubricin expression (Sun et al. 2006) and that mechanical deformation of fibroblasts and the ECM in tendons have effects on its microstructure, composition and cellularity (Gillard et al. 1979).

In summary, ESWT was found to stimulate endogenous lubricin production in tendons and septa in the rat hindlimb, reflected in the percentage of cells containing lubricin and the percentage of the ECM displaying the presence of the mucinous glycoprotein. A form of mechanical loading, ESWT stimulates the upregulation of lubricin, pointing to the fact that this form of physical stimulus induces excessive expression of lubricin in vivo. This result provides a basis for the hypothesis that increased lubricin deposition in tendons and septa following ESWT contributes to the beneficial effects of ESWT by facilitating movement macroscopically among gross structures, as well as microscopically among collagen fascicles. Lubricin may decrease wear and tear in tissues treated with ESWT and thus provides relief from symptoms and pain.

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