Local trauma in human patellar tendon leads to a widespread increase in tendon cell activity

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Abstract (250 words max)

Low cellular activity and slow tissue turnover in human tendon may prolong resolution of tendinopathy. This may be stimulated by moderate localized traumas such as needle penetrations, but whether this results in a widespread cellular response in tendons is unknown. In an initial hypothesis-generating study, a trauma-induced tendon cell activity (increased total RNA and collagen I mRNA) was observed after repeated patellar tendon biopsies in young men. In a subsequent controlled study, 25 young men were treated with two 21-gauge needle penetrations (n=13, needle group (NG)) or one 14-gauge needle biopsy (n=12, biopsy group (BG)) in one patellar tendon. Four weeks later biopsies were taken from treated (5 mm lateral from trauma site) and contralateral tendons for analyses of RNA content (ribogreen assay), DNA content (PCR based), and gene expression for relevant target genes (Real-time RT-PCR) (NG, n=11 and BG, n=8). Intervention increased RNA content, and mRNA expression of collagen I and -III and TGF-ß1 ($p<0.05$), with biopsy treatment having greatest effect (tendency for RNA and collagen I). Results for DNA content were inconclusive, and no changes were detected in expression of Insulin like growth factor-I, connective tissue growth factor, scleraxis, decorin, fibromodulin, tenascin-C, tenomodulin, VEGFa, CD68, IL-6, MMP12 and MMP13. In conclusion, a moderate trauma to a healthy human tendon (e.g. biopsy sampling) results in a widespread up-regulation of tendon cell activity and their matrix protein expression. The findings have implications for design of studies on human tendon, and may provide perspectives in future treatment strategies towards tendinopathic conditions.

Introduction

Tendons connect muscle to bone and pain-free function of tendon tissue is essential in voluntary movements. In healthy adult tendon the bulk of the tendon matrix has very slow turnover (18, 33, 40), and the activity of tendon fibroblasts also appears limited, as very low concentrations of total RNA are found in tendon tissue (17, 21, 22, 42). Even in chronic Achilles tendinopathy a low cell activity seems to be maintained, as the RNA content is not increased compared to healthy tendon ((21) and own unpublished observation). The limited RNA concentration and the very slow tissue turnover in adult human tendon (17, 18), suggest that tendon fibroblasts are in a dormant state, and this may inhibit the resolution of e.g. overuse tendinopathy. On the other hand acute tendon rupture induces cell activity and results in up-regulation of type I collagen expression in humans (23), and a similar response is seen animals after tendon transection (14, 31). The dramatic healing response after tendon rupture is underlined by the observation that even non-surgical treatment of Achilles tendon rupture in adult humans can result in regeneration and regaining of tendon function (1). In addition, it is known that primary fibroblasts derived from healthy, adult human tendon can be activated to form new tendon-like tissue in culture (3). In combination, these observations suggest a marked capacity for cellular activation and matrix protein synthesis in tendon in response to a trauma. Therefore a controlled trauma may be useful for initiating a cellular response in tendon, and could potentially accelerate cell activity in e.g. chronic tendinopathy.

Procedures of thin needle penetration of tendon, known as dry needling, have been used as an attempt to cure chronic pathological tendon conditions (tendinopathy), and have been shown in some studies to be clinically effective in different tendons (26, 30, 37, 38). However, the outcome

measures of these trials have been mainly clinical, and apparently no attempts have been performed to evaluate the influence of needle penetration on tendon cell activity or matrix protein expression. Preliminary evidence does suggests that trauma induced by biopsy procedures in human tendon tissue may lead to an activation of tendon cells lasting for several months (12), but this has not been investigated in controlled studies.

This article includes two separate experiments involving the human patellar tendon. The first study was a mainly hypothesis-generating study that suggested a potentially large effect of tendon biopsy procedures on tendon cell activity. This study was followed up by a second well-controlled experiment that systematically investigated the effect of both mild and moderate trauma to tendon tissue. The specific aim of the second study was to test whether needle penetration (mild trauma) and biopsy sampling (moderate trauma) in an isolated part of the tendon could lead to a widespread activation of the tendon cells in healthy patellar tendons of young men. We hypothesized that tendon human fibroblasts would respond to acute tissue trauma by increasing cellular activity and gene expression of matrix proteins, and that the effect of the moderate trauma would be larger than that of the mild trauma.

Materials and Methods

Hypothesis-generating study (study I)

An experiment was set up to study the effect of three weeks of unloading (unilateral lower limb suspension (ULLS)), followed by three weeks of resistance training, on patellar tendon and skeletal muscle in young men (7). Data from the muscle biopsies obtained in this study have been published previously, and details regarding subject characteristics, ethical permissions and the ULLS- and resistance training (RT) interventions can be found here (7). With the purpose of

studying the tendon tissue response to unloading and reloading, three patellar tendon biopsies were obtained; one at baseline (pre-biopsy), one after ULLS (post ULLS) and one after resistance training (post RT). In the first half of the participants (n=5) all three biopsies were obtained from the leg that was subjected to ULLS. In the remaining persons (n=4) the pre-biopsy was taken in the contra-lateral leg (not subjected to ULLS), while the post ULLS and post RT biopsies were taken from the leg that was subjected to ULLS. Due to a drop-out, only 8 samples were available at the post RT time-point. The biopsies were obtained according to the method previously described (17). Briefly, the skin was anesthetized with Lidocaine, and the biopsy was taken from the proximal part of the patellar tendon with automatic disposable 14 G Bard Monopty Biopsy Instrument (Bard limited, Crawley, UK) at an angle of 35–45°.

The outcome of this study suggested that the first biopsy led to a traumatic response in the tendon that affected the RNA content and collagen I mRNA expression in tendon tissue obtained from the repeated biopsies (Fig. 1). This effect outweighed any potential effect of unloading and resistance training, and thus made the data unusable for studying unloading and resistance training in itself. However, the observations generated the hypothesis that a trauma to tendon tissue may activate tendon cells, perhaps even far from the site of the trauma. To investigate this in a more systematic way, study II was conducted.

Main study (study II)

The main study tested the effect of two different types of trauma on the patellar tendon. One treatment consisted of two thin needle penetrations and the other of a biopsy procedure. Four weeks after the initial treatment, tissue biopsies from both the treated and non-treated patellar tendons were obtained (Fig. 2), and these tissue samples were analyzed to assess the effect of the

two types of trauma. In this study great care was taken not to enter the tissue in the same location as the initial trauma, in order to specifically investigate the effect of a local trauma on the global response in the tendon tissue.

Participants and samples numbers study II

Twenty-five moderately active healthy young men were included in study II. Exclusion criteria were; smoking, body mass index higher than 28, systemic disease, disease involving connective tissue and joints, substance abuse, and prior trauma to the patellar tendon. The participants were randomized into a needle treatment group (n=13) and a biopsy treatment group (n=12). However, four participants dropped out before the study was finalized (three failed to show up for the second appointment and one participant sustained a knee injury unrelated to the study), and additionally two samples were lost during analyses. The data are therefore based on paired samples (control vs. treated leg) of n=11 for the needle group and n=8 for the biopsy group. There were no differences between the needle and biopsy groups with respect to age (24 ± 2.2 vs. 23 ± 2.4 (mean ± SD)) or body mass index (BMI) (23±1.6 vs. 23±1.5 (mean ± SD)). All subjects gave informed consent, and the study was approved by the Ethical Committee of the Capital Region of Denmark (H-4-2012-152). The study was conducted in accordance with the Helsinki Declaration.

Biopsy and dry needling procedures study II

The biopsy and dry needling procedures were both carried out on the patellar tendon. The point of entry for both was close to the mid-proximal region, and slightly to the medial side of the midline (to leave space to obtain a biopsy 4 weeks later from an area untouched by the initial trauma) (Fig. 2). The overlying skin was anesthetized with 2-3 ml Lidocaine (1%) and the area was

147 sterilized. A 5-mm long incision (proximal-distal direction) in the skin was made down to the
148 surface of the tendon prior to both the biopsy and needle treatment procedures.

149 The biopsy procedure was performed in one patellar tendon on all participants in the biopsy group
150 (Fig. 2B). The sample was obtained with ultrasound guidance using a 14-gauge (2.1 mm diameter)
151 disposable core tissue biopsy instrument (Bard Magnum, cat. no: MN1410) used with a Bard
152 Magnum Biopsy instrument (Bard Magnum Biopsy Instrument; CR Bard, Covington, GA) as
153 described previously (12)

154 The needling procedure was performed in one patellar tendon of all participants in the needle
155 group, and consisted of two ultrasonographically guided penetrations into the tendon using a 21-
156 gauge (0.8 mm diameter) needle (KD Medical, Berlin, Germany) (Fig. 2A). The needle penetration
157 went as deeply as the biopsy needle, and that the same angle, and the two needle penetrations
158 were spaced 1-2 mm apart.

159 Four weeks after the initial treatment, post-biopsies were obtained from both patellar tendons
160 (treated and control tendon) in all participants. The biopsies were obtained as described above,
161 the only difference being that the post-treatment biopsies were obtained laterally to the midline
162 of the tendon, leaving at least 5 mm between the initial medially located trauma and the post-
163 biopsies. The distance between the initial trauma and the post-biopsies was ensured both by
164 visual confirmation of the first skin incision and by ultrasound (Fig. 2).

165 Tendon biopsy samples were rinsed with saline to remove any blood, and then tendon and fat
166 tissue were separated under a microscope. After removing excess saline both tendon and fat
167 fractions were frozen in liquid nitrogen and stored at -80 °C for later analyses.

RNA extraction (study I and II)

All tissue samples were weighed before RNA extraction. The tendon samples weighed 8-22 mg in study I and 3-15 mg in study II. The fat fractions from study II weighed 0.7-7 mg (all wet weights). For study I tendon tissue was homogenized in 1 ml of TriReagent (Molecular Research Center, Cincinnati, OH, USA) containing five stainless steel balls of 2.3 mm in diameter (BioSpec Products, Inc., Bartlesville, Oklahoma, USA), and five silicon-carbide sharp particle of 1 mm (BioSpec Products, Inc.), by shaking in a FastPrep®-24 instrument (MP Biomedicals, Inc., Illkirch, France) at speed level 4 for 15 s. The shaking step was repeated five times with cooling on ice between each shaking step (to avoid heating of the sample). A similar homogenization procedure was used in study II. However only one silicon-carbide sharp particle was used and the samples were only shaken once in the FastPrep®-24 instrument (this has been found to reduce the loss and increase the quality of RNA, compared to the procedure used for study I)

Following homogenization, bromo-chloropropane (Molecular Research Center, Cincinnati, OH, USA) was added in order to separate the samples into an RNA containing aqueous and an organic phase with DNA in the inter phase. Following isolation of the aqueous phase (the remaining phases used for DNA extraction), glycogen was added (80 µg) to improve RNA precipitation. RNA was precipitated using isopropanol and the resulting pellet was washed in 75% ethanol, re-solubilized in 100 µl RNase-free water, re-precipitated with 1/10 vol 3 M sodium acetate pH 5.5 and 2 vol 96% ethanol, washed in 75 % ethanol and finally dissolved in 10 µl RNase-free water. Total RNA was quantified with RiboGreen assay (R-11490, Molecular Probes, OR, USA).

Real time RT PCR (study I and II)

For study I, 1 µl of the extracted RNA was used for cDNA synthesis, equal volume chosen due to the high variation in yield. However, cDNA synthesis based on equal RNA amount was also performed (data not shown) and this did not change the conclusions made. But for study II 50 ng of RNA from the tendon samples was used for cDNA synthesis. As the RNA yield from the fat tissue was too low for reliable quantification 5 µl of the extracted RNA from fat was used for cDNA synthesis.

RNA was converted into cDNA in 20 µl using OmniScript reverse transcriptase (Qiagen, California, USA, and 10 mM poly-dT (Invitrogen, Naerum, Denmark) according to the manufacture's protocol (Qiagen). For each target mRNA, 0.25 µl cDNA was amplified in a 25 µl SYBR Green polymerase chain reaction (PCR) containing 1 x Quantitect SYBR Green Master Mix (Qiagen) and 100 nM of each primer (Table 1). The Ct values were related to a standard curve made with DNA oligomers matching the target sequence (Ultramer™ Oligos, Integrated DNA Technologies, Inc., Leuven, Belgium) in order to determine the relative difference between the unknown samples, accounting for the PCR efficiency. The specificity of the PCR products was confirmed by melting curve analysis after amplification. The large ribosomal protein P0 (RPLP0) was chosen as internal control, as RPLP0 mRNA has been suggested to be constitutively expressed (11). To validate this assumption, another unrelated "constitutive" RNA, GAPDH mRNA, was measured and RPLP0was normalized to GAPDH. No significant effect was seen of the interventions on the expression of GAPDH relative to RPLP0 (Fig. 1B and Fig. 3).

DNA extraction and quantification (study II)

DNA was extracted according to the alternative DNA isolation procedure provided in the Tri-reagent protocol (Molecular Research Center, Cincinnati, OH, USA) with a few modifications. 0.5 ml DNA extraction buffer (4 M guanidine thiocyanate, 50 mM sodium citrate, 1 M Tris (free base), 100 ug/ml salmons testes DNA (D-7656, Sigma, St. Louis, MO, USA)) was added to the interphase - organic phase mixture that was left over from the RNA extraction, mixed, heated at 50°C for 15 min, mixed and left at room temperature for 30 min. The water phase containing DNA was isolated and mixed with 6 ul polyacryl carrier (Molecular Research Center, Cincinnati, OH, USA). DNA was precipitated with isopropanol, washed with 75% ethanol and resuspended in 100 µl 8 mM NaOH. The DNA was diluted 100 times in TE pH8 and 5 µl used per 25 µl PCR reactions in the same way as for the cDNA targets using primers for the myogenin promoter (AGGTGCTGTCAGGAAGCAAGGA, TAGGGGGAGGAGGGAACAAGGA).

Statistics

For study I no statistical tests were performed, as this was considered only as a hypothesis-generating study. For study II all data were log-transformed before statistical analyses and are presented as geometric means +/- back-transformed SE. A two-way repeated measures analysis of variance (ANOVA) on leg (control vs. treated)*group (needle vs. biopsy) was performed. If the two-way ANOVA was significant, individual differences between control- and treated tendons, within treatment groups, and differences between treatment groups within control and treated tendons were tested with a post-hoc test (Student-Newman-Keuls). Otherwise, main effects of treatment (control vs. treated) and group (needle vs. biopsy) were tested. Differences were considered significant when $p < 0.05$. All statistical analyses were performed with SigmaPlot. Mean

values for the pre-biopsy samples are included in graphs for study II, but data for pre-biopsies are not included in statistical tests.

Results

Results from study I

The analyses of tendon biopsies from study I indicated that the tendon tissue was affected by the trauma created by the first biopsy. The RNA content in first biopsies ranged from 30-100 ng/mg tissue, while repeated biopsies contained 58-556 ng/mg tissue (Fig. 1A), showing that a prior biopsy led to an increased content of total RNA in the tissue. In addition, the COL1A1 mRNA expression was clearly increased in the repeated biopsies compared to first biopsies, and in some samples the expression level was more than 50-fold higher in the repeated biopsies than in naïve tissue (Fig. 1 C). GAPDH mRNA appeared stable and no obvious effect of on scleraxis was seen (Fig. 1B,D).

Total RNA and DNA concentration (study II)

In study II pre-biopsies and biopsies from control tendons contained mean values of 36-40 (range of individual values 18-100) ng RNA/mg tissue, while the mean level of RNA was 43 (range 20-75) ng/mg after needle treatment and 57 (range 40-86) ng/mg after the biopsy treatment (Fig. 4A). The concentration in treated samples was significantly higher than in control samples (main effect of treatment) (Fig. 4A). The ratios between treated and control leg (within-subjects) in the needle and biopsy groups suggest that the significant main effect of treatment is mostly driven by an increase in the RNA concentration in the biopsy treatment group (top right inset graph in Fig. 4A) and there was a trend for a leg*treatment interaction ($p = 0.081$).

For DNA, mean values of 124-144 ng DNA/mg tendon tissue were found in pre-biopsies and control tendons, while the mean level of DNA was 134 ng/mg after needle treatment and 180

ng/mg after the biopsy treatment (Fig. 4B). However, no significant changes were detected, although there was a trend for a leg*treatment interaction ($p = 0.068$).

When comparing ratios of RNA to DNA (Fig. 4C) these were quite stable and no significant changes were found.

Expression of mRNA for matrix proteins (study II)

The expression pattern of collagen I and -III were very similar and showed a relatively robust increase in expression in response to the biopsy treatment, while there was no obvious effect of the needle procedure (Fig. 5A & 5B). For COL3A1 there was a clear leg*group interaction and the expression level was higher in the biopsy treated leg compared to both the biopsy control leg and the needle treated leg. For COL1A1 there was only tendency for a leg*group interaction ($p=0.053$), but a main effect of treatment was clear (Fig. 5A). However, it is assumed that collagen I mRNA expression follows the same pattern as collagen III mRNA because of a strong correlation between the two ($r^2 = 0.950$, $p<0.001$), and that the biopsy procedure induced type I collagen, while the needle procedure likely did not.

No changes were seen for collagen XII expression (Fig. 5C), while fibronectin had a generally higher level of expression in the biopsy group (Fig. 5D). Expression of decorin was relatively high and stable and was found to be unaffected by the interventions (Fig. 6). Fibromodulin, tenascin-C and tenomodulin were expressed at relatively low and variable levels, and no significant changes could be detected (Fig. 6).

Expression of mRNA for collagen inducing factors (study II)

Expression of all three isoforms of transforming growth factor-ß (TGF-ß) were measured due to their known collagen inducing action (20) (Fig. 7A,B,C). TGF-ß1 had an expression pattern similar

to collagen I and –III, with a significant increase in expression level in the biopsy treated leg compared to both the biopsy control leg and the needle treated leg (Fig. 7A). The variation in both TGF-ß2 and TGF-ß3 expression levels was relatively high and potential treatment-induced changes may be difficult to detect considering the relatively low number of subjects. Similar considerations may be relevant for scleraxis, where no significant changes were detected (Fig. 7F). IGF-IEa and CTGF, which are also known to induce collagen expression (6, 12, 13, 16), showed relatively stable levels of expression and no effects of needle or biopsy treatment were found for these growth factors (Fig. 7D, E).

Inflammatory markers (study II)

Expression levels of vascular endothelial growth factor a (VEGFa), interleukin-6 (IL-6) and CD68 (macrophage marker) were measured to detect potential signs of neo-vascularization and inflammation/macrophage infiltration (IL-6/CD68), but no significant changes were found (Fig. 8). The level of IL-6 mRNA expression was extremely low in both control and treated samples, and the large degree of variation for this target can be explained by stochastic variation (Fig. 8A). VEGFa expression was also low, but relatively stable (Fig. 8B) and CD68 was expressed at relatively high and stable levels (Fig. 8C).

Transcripts with low expression levels (study II)

Levels of MMP13, MMP12, myostatin, Ki67 and TNFα mRNA were very low and undetectable in many of the samples, and therefore data have not been included.

Expression of mRNA targets in fat (study II)

RNA concentrations were very low and variable in the fat samples. Therefore we chose to make pools of fat RNA and screen these for expression of GAPDH, RPLP0, COL1A1, COL1A3, COL12A1,

298 IGF-IEa, CTGF, TNFα, fibromodulin, fibronectin, tenomodulin, MMP13, tenascin-C, TGF-ß1, TGF-ß2,
299 TGF-ß3, IL6, Ki67 and VEGFa. The expression pattern for these targets was very similar to the
300 expression pattern seen in the tendon RNA samples, suggesting that the RNA extracted from the
301 fat samples consisted mainly of tendon RNA contaminating the fat RNA. Based on this finding we
302 chose not to make further analyses on the fat RNA samples.

303

Discussion

The main finding of the present study is that a moderate trauma into a healthy human tendon results in an increased total RNA content and an increased expression of the major types of tendon collagens, even in tendon areas separated from the site of trauma. This suggests a widespread activation of the resident tendon fibroblasts in response to a localized trauma, and such a response may explain the positive effect of tendinopathy treatments that involve a controlled trauma such as dry needling (26).

The bulk of tendon collagen has a slow turnover in adults (18, 33, 40), and the low content of total RNA found in tendon tissue (17, 21, 22, 42) suggests that the activity of tendon fibroblasts is also limited. It may be speculated that a low cell activity and slow tissue turnover is advantageous when the tendon is healthy, but could inhibit healing when the tissue is affected by overuse of the tendon. Based on this theory, the beneficial effect of dry needling may relate to a trauma-induced activation of tendon fibroblast that results in tissue regeneration. However, the insight into the tendon response to trauma on a cell/molecular level is very limited.

Tendon tissue biopsies lead to increased cell activity

The present article is based on results from two separate studies. First, a study was performed to examine the effect of tendon unloading, in which consecutive biopsies were taken from the same patellar tendon in healthy young men. From this experiment it became clear that the tissue sampled in the second and third biopsies from the same tendon was markedly affected by the trauma created by the preceding biopsy(s). This precluded using the tissue for evaluating the effect of unloading, but also generated the hypothesis that a local trauma in tendon tissue may

activate tendon cells, perhaps even far from the site of the trauma. The effect of the repeated biopsies included an increase in total RNA content of up to 7 fold as well as marked increases in the mRNA expression of type I collagen (up to 50 fold, but variable). Since ribosomal RNA makes up the bulk of total RNA, the rise in RNA indicates a higher content of ribosomes and thus translational potential/activity of the cells contained in the tissue biopsy. The rise in collagen I expression suggests that this increased translational activity occurs due an increased activity and/or proliferation of the local collagen-producing tendon fibroblast, as these cells are known to produce high levels of collagen I mRNA (3). However, these results were preliminary and could not establish whether or not there was any increase in cell number and whether invading cells, such as macrophages, were perhaps partly responsible for the increased cell activity. Also the distance between first and repeated biopsies was not well controlled and therefore it was uncertain how far the effect of the trauma spread through the tendon.

To investigate these questions, a second experiment was performed that systematically examined the effect of localized trauma (both mild and moderate) on cell activity (total RNA), cell number (DNA content) and gene expression in healthy patellar tendon tissue located at least 5 mm from the site of the trauma. An increase in total RNA content in response to treatment was found, indicating the cell activity was increased in a relatively wide range from the tissue trauma, though the increase was not as dramatic as that seen in the hypothesis-generating study (Fig. 1 & Fig. 4). This discrepancy may relate to the fact that the minimum distance between first and repeated biopsies was under better control in this second study, and the most dramatic increases seen in the first study could be explained by sampling of tissue from or very close to the actual damage site (Fig. 2).

Previous studies on tendon injury have mainly focused on the actual damaged/healing tissue and consequently very little data exist with regard to the response of the untouched tissue in the vicinity of the damage site. Therefore, to our knowledge, no published data are directly comparable to the present data. However, one study on humans indicated a possible increase in RNA content in ruptured tendon (22), and a study on rabbits showed a 6-fold increase in total RNA in response to transection and re-suturing of flexor tendon (4). The large increase in total RNA in the rabbit tendon compared to our results may relate to the species difference, the severity of the injury, and the fact that the analyzed rabbit tendon tissue included the actual damage site.

The DNA content was measured in order to assess the cell content and evaluate whether the treatments induced either cell proliferation and/or cell invasion. No significant effects of the treatments were found, but considering the relatively large variation in the measured DNA levels, combined with the low sample number (due to drop-outs), it is problematic to conclude that no changes occurs in cell number in response to the treatment. In relation to this it should be noted that there was a trend towards interaction between leg and treatment ($p = 0.068$, Fig. 4), and it may be speculated that the DNA concentration was affected by the biopsy treatment, but that this was not detectable due to high variance and low sample number. Previous studies using collagenase treatment to induce tendon injury in horses and rabbits have shown robust increases in DNA in the injured tissue (10, 34), but these studies are not easily compared to the present data due to injury severity and sampling site.

In summary, it is evident that the localized trauma leads to an increased cell activity even in a

distance of minimum 5 mm from the trauma in tendon tissue, but it is not possible based on the present data to conclude whether an increase in cell number is partly responsible for the increase in cell activity.

Increased expression of collagen and TGF-ß1 after biopsy treatment

With regard to changes in expression of specific mRNA targets in response to the needle and biopsy treatments, the most robust changes were seen in type I and III collagen. It was clear that the biopsy treatment, but not the needle treatment, led to increased collagen III expression, and a very similar picture was seen for type I collagen (Fig 5A & B). The induced expression of collagen I and III suggests that the local tendon fibroblasts are activated by the biopsy treatment, as such an expression profile would not be expected if the increase in cell activity (total RNA increase) was mainly caused by an invasion of immune cells. To further investigate a possible immune cell response, CD68 was measured, as this gene known to be expressed at high levels in macrophages (19). However, the level of CD68 expression was unaffected by the treatment (Fig. 8C) and was in fact high in both control and treated tendon tissue (absolute values not shown). The level of CD68 expression corresponded to that of e.g. fibromodulin and CTGF (though a direct comparison of absolute values is not appropriate to make due to potential variation in the efficiency of the cDNA synthesis for different targets), and notably the level of CD68 expression was far higher than what we have observed in human skeletal muscle (personal observation). This observation, combined with a previous study showing that fibroblasts express relatively high levels of CD68 (15), indicates that perhaps the tendon fibroblasts, and not macrophages, are the major source of CD68 expression in the tendon samples. An almost absent expression of MMP12, a proteinase that is also expressed at high levels in macrophages (43), was found in both control and treated tendon, and this supports that no large influx of macrophages was induced by the treatment. This

speculation is further supported by the fact that at least no dramatic increase occurred in DNA content.

With regard to the other mRNA targets related to the structural tendon matrix, no changes were seen in decorin, tenascin-C, fibromodulin and tenomodulin, while fibronectin had a generally higher level of expression in the biopsy group. It may be suspected that the main effect on fibronectin is largely caused by an increased expression in response to the biopsy treatment, but this cannot be concluded by the present data. However, an effect of the biopsy treatment on both collagen (I and III) and fibronectin would fit theoretically with the evident induction of TGF-ß1 seen in response to the biopsy treatment (Fig. 7A), since TGF-ß1 is known to induce both collagen and fibronectin expression (20, 25, 32, 44). Several studies on severe injury in animal tendons have found substantial increases in expression levels of collagen I and/or collagen III (4, 10, 14, 31) as well as in TGF-ß1 (8, 10, 14, 39) in the damaged tissue compared to control tendon. In addition, one study in humans found elevated type I collagen expression in ruptured Achilles tendon tissue (23). The present study adds information to these observations by showing that even a moderate trauma can lead to increased TGF-ß1 expression along with collagen I and III expression, and that this response is not restricted to the actual damaged tissue but spreads out through the tendon and lasts for several weeks.

An increase in TGF-ß1 action may well be the cause of the found increase in collagen expression, but other growth factors, such as IGF-I and CTGF, as well as the transcription factor scleraxis may also be involved. Both IGF-I and CTGF have been suggested to be part of the tendon healing response and both are known to induce collagen expression (6, 12, 13, 16). The transcription factor scleraxis is known to be involved in tendon development and to induce collagen I

416 expression (9, 28). However, we observed no differences in the expression level of either IGF-I,
417 CTGF or scleraxis in treated versus control tendon.
418
419 With regard to IGF-I, this observation differs from previous studies on animals (rabbit, horse and
420 chicken) in which increased levels of IGF-I expression were seen in response to severe tendon
421 damage (8, 39), and in some studies this elevation persisted even at late time points possibly
422 corresponding to the one we have investigated (5, 10). Many potential explanations for this
423 divergence exist, including severity of the injury, species difference and timing of tissue sampling.
424 With regard to the CTGF response to tendon injury, two studies on chick flexor tendon showed an
425 increase in expression shortly after tendon transection (3-7 days) (8, 39), while a similar
426 experiment in rabbit flexor tendon showed unaltered CTGF expression in the early healing phase
427 and a down-regulation in the late healing phase (24 days) (4). The decrease in CTGF in the late
428 healing phase is supported by the data on chick tendon from Chen et al. (8). Based on these
429 previous observations it does not seem surprising that CTGF expression was unaffected at 4 weeks
430 after treatment, although it cannot be excluded that a change occurred at an earlier time point.
431 Previous studies of scleraxis expression in healing tendon show variable results, with some studies
432 showing an increase in expression in response to tendon injury (24) and others finding no change
433 (14, 41).
434
435 In summary, the biopsy treatment induced an expression of both type I and III collagen that
436 persisted for 4 weeks after treatment, and based on the expression of collagen-inducing growth
437 factors it seems likely that an increased TGF-ß1 action is - at least partly - involved in the induction
438 of collagen mRNA expression at this time point, while CTGF and IGF-I seem less likely to be

involved.

VEGF expression

VEGF expression was measured in control and treated tendon to assess a potential angiogenic response to the induced trauma, but no effect was detected at 4 weeks after the trauma (Fig. 8B). In adult human healthy tendons the VEGF expression on both mRNA and protein level is suggested to be very low or absent, while it is expressed in both fetal tendon and ruptured adult tendon tissue (36). In addition, previous studies of tendon injury and healing of chick flexor tendon show substantial increases in VEGF mRNA in the early healing phase (3-7 days) but at later time points the expression level was normalized (8, 39). Thus, trauma in tendon tissue seems a potent stimulus for inducing VEGF expression, but our data suggest that either this effect does not spread out from the site of the trauma or that the effect is short lasting also in human tendon (< 4 weeks).

The tendon response to needle and biopsy treatment

There was a clearly more pronounced effect of the biopsy treatment than of the needle treatment on type III collagen and TGF-ß1 expression, and though only a main effect of treatment was found for the total RNA content and type I collagen expression, this was most likely driven by the effect of the biopsy treatment ($p=0.081$ (RNA) $p= 0.053$ (collagen I) for interaction, Fig. 4 & 5). Therefore it is unlikely there was any effect the needle treatment at all. The greater effect of the biopsy treatment may be related to the fact that the thick biopsy needle cuts through the collagen fascicles in the site of penetration, while it may be suspected that the thin needle could penetrate in between fascicles and thus no or minimal cutting would occur. The cutting of fibrils is very likely to generate a marked response in the tendon cells due to the sudden unloading of the matrix and thereby the cells (2, 27) and evidently this response is propagated to the surrounding tissue, as the

treatment response was observed in an area separated from the actual injury site. The spreading of the response fits well with previous evidence of gap junctions between tendon cells that allow communication between them in both lateral and vertical directions (29). Whether the observed response is favorable in relation to tendon healing is questionable. The general increase in cell activity could promote healing, and such cell activation may explain why needle penetrations (5-50 penetrations) of tendinopathic tendons seem beneficial in some cases (26, 30, 37, 38). On the other hand the gene expression profile with high TGF-ß1, along with high type I and III collagen expression (and possibly fibronectin) is partly similar to what is seen in tendinopathic tendon (35), although we did not observe changes in tenascin-C, fibromodulin or decorin quite corresponding to that observed in tendinopathy (35). Furthermore, we saw no up-regulation of MMP13, an enzyme that is induced by tendon fascicle damage and/or unloading (27), though studies in vitro on rat tail tendon showed that the response was localized to cells located in the site of damage (27).

Limitations

One limitation of the study is that it is only possible to measure the effect of the trauma at one time-point in each person. Due to this limitation we cannot determine a time-line with regard to changes in expression of relevant genes, and thus we cannot rule out that the seemingly un-responsive target genes actually responded at earlier or later time-points than where our observations are made. An additional limitation is the relatively low number of subjects due to unforeseeable dropouts. This low number reduces the confidence with which we can conclude that there was no effect of treatment, especially for the parameters that showed high levels of variation. These parameters include DNA content, and expression levels of TGF-ß2, TGF-ß3, fibromodulin, tenascin-C and tenomodulin.

Conclusion

In conclusion tendon cells can be activated by trauma, and this activation seems to be based on an increased activity, and perhaps proliferation, of the local tendon cells. Importantly, the response is not restricted to the actual damage site but propagates at least 5 mm from the sites of damage. As tendons have relatively low tissue turnover and cell activity, and show poor resolution of tendinopathic conditions, the present findings of increase in cell activity after trauma could explain a potential beneficial effect of dry needling in the treatment of tendinopathy. Finally, the findings have implications for design of studies with tendon biopsy sampling, as they clearly show that it is not feasible to perform repeated biopsy sampling of tendon tissue from the same tissue, unless the aim is to study the specific effect of trauma itself.

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Figure legends

Figure 1

RNA content (A) and mRNA expression for GAPDH (B), COL1A1 (C) and Scleraxis (D) was measured in patellar tendon tissue before the intervention (pre), after three weeks of unilateral limb suspension (post ULLS) and after three subsequent weeks of resistance training (post RT). mRNA levels were normalized to RPLP0 mRNA. Data are shown as individual values for first biopsies (open circles) and repeated biopsies (filled circles). In the first half of the participants (n=5) all three biopsies were obtained from the leg that was subjected to ULLS and therefore these persons had one first- and two repeated biopsies. In the remaining persons (n=4) the pre-biopsy was taken in the contra-lateral leg (not subjected to ULLS), while the post ULLS and post RT biopsies were taken from the leg that was subjected to ULLS, and therefore these persons have two first- and one repeated biopsy. At the pre time-point the scleraxis mRNA level was below the detection limit in three samples (D).

Figure 2

Illustration of the interventions in study II. Participants in the needle group were subjected to two penetrations of the patellar tendon with a 21-gauge needle on the first day of the study (A), while participants in the biopsy group had one biopsy taken from the patellar tendon (B). For both groups this initial trauma was localized slightly to the medial side of the tendon. After 4 weeks, all participants had biopsies taken from both the treated and the control tendon. These biopsies were taken slightly to the lateral side of the tendon and at least 5 mm from the initial site of trauma (initial trauma shown by short black line on treated tendon).

Figure 3

Tendon GAPDH mRNA normalized to RPLP0 mRNA, presented as fold changes relative to the mean of control values from both needle and biopsy groups. Values are geometric means ± SE. Mean values for the ratio between control and treated leg (within subjects) for the needle and biopsy groups are shown in small inset graphs (top right). P-values for the two way repeated measures ANOVA are shown (top left).

Figure 4

Concentrations of A) RNA and B) DNA (per tissue wet-weight) and C) RNA/DNA ratios, presented as fold changes relative to the mean of control values from both needle and biopsy groups. Values are geometric means ± SE. A value of 1 corresponds to approx. 38 ng RNA/mg tissue and 135 ng DNA/mg tissue. Mean values for the ratio between control and treated leg (within subjects) for the needle and biopsy groups are shown in small inset graphs (top right). The RNA concentration was higher in treated versus controls (main effect of leg) (# $p<0.05$). P-values for the two way repeated measures ANOVA are shown, with bold text indicating significant p-values (lower left).

Figure 5

Tendon COL1A1, COL3A1, COL12A1 and fibronectin mRNA normalized to RPLP0 mRNA, presented as fold changes relative to the mean of control values from both needle and biopsy groups. Values are geometric means ± SE. Mean values for the ratio between control and treated leg (within subjects) for the needle and biopsy groups are shown in small inset graphs (top right). COL1A1 expression was higher in treated versus controls (main effect of leg) (# $p<0.05$). COL3A1 expression was higher in biopsy treated compared to both biopsy control and needle treated (*** $p<0.001$). Fibronectin had higher expression levels in the biopsy group (main effect of group) ($

p<0.05). P-values for the two way repeated measures ANOVA are shown, with bold text highlighting significant p-values (top left).

Figure 6

Tendon decorin, fibromodulin, tenascin-C and tenomodulin mRNA normalized to RPLP0 mRNA, presented as fold changes relative to the mean of control values from both needle and biopsy groups. Values are geometric means ± SE. Mean values for the ratio between control and treated leg (within subjects) for the needle and biopsy groups are shown in small inset graphs (top right). P-values for the two way repeated measures ANOVA are shown, with bold text highlighting significant p-values (top left).

Figure 7

Tendon TGF-ß1, TGF-ß2, TGF-ß3, CTGF, IGF-IEa and scleraxis mRNA normalized to RPLP0 mRNA, presented as fold changes relative to the mean of control values from both needle and biopsy groups. Values are geometric means ± SE. Mean values for the ratio between control and treated leg (within subjects) for the needle and biopsy groups are shown in small inset graphs (top right). TGF-ß1 expression was higher in biopsy treated compared to biopsy control (***p<0.001) and needle treated (*p<0.05). P-values for the two way repeated measures ANOVA are shown, with bold text highlighting significant p-values (top left).

Figure 8

Tendon VEGFa, IL-6 and CD68 mRNA normalized to RPLP0 mRNA, presented as fold changes relative to the mean of control values from both needle and biopsy groups. Values are geometric means ± SE. Mean values for the ratio between control and treated leg (within subjects) for the

706 needle and biopsy groups are shown in small inset graphs (top right). P-values for the two way

707 repeated measures ANOVA are shown (top left).

708

709

710 Table 1

mRNA Target	Sense Primer	Anti-sense primer	Accession No.
CD68	CAGCTTTGGATTCATGCAGGACC	CTCTGCCCCAGGGGTGCTTG	NM_001040059.1
Collagen I (COL1A1)	GGCAACAGCCGCTTCACCTAC	GCGGGAGGACTTGGTGGTTTT	NM_000088.3
Collagen III (COL3A1)	CACGGAAACACTGGTGGACAGATT	ATGCCAGCTGCACATCAAGGAC	NM_000090.3
Collagen XII (COL12A1)	CCCAGGTCCTCCTGGATACTGTGA	GCAGCACTGGCGACTTAGAAAATGT	NM_004370.5
Connective tissue growth factor (CTGF)	TGCGAAGCTGACCTGGAAGAGA	GCCGTCGGTACATACTCCACAGAA	NM_001901.2
Decorin (DCN)	GGTGGGCTGGCAGAGCATAAGT	TGTCCAGGTGGGCAGAAGTCA	NM_001920.3
Fibromodulin (FMOD)	CAGTCAACACCAACCTGGAGAACC	TGCAGAAGCTGCTGATGGAGAA	NM_002023.4
Fibronectin (FN1)	TTTGCTCCTGCACATGCTTT	TAGTGCCTTCGGGACTGGGTTC	NM_212482.1
GAPDH	CCTCCTGCACCACCAACTGCTT	GAGGGGCCATCCACAGTCTTCT	NM_002046.5
Insulin like growth factor-I (IGF1-Ea)	GACATGCCCAAGACCCAGAAGGA	CGGTGGCATGTCACTCTTCACTC	NM_001111284.1
Interleukin-6 (IL6)	GAGGCACTGGCAGAAAACAACC	CCTCAAACTCCAAAAGACCAGTGATG	NM_000600.3
Ki-67 (MKI67)	CGGAAGAGCTGAACAGCAACGA	GCGTCTGGAGCGCAGGGATA	NM_002417.4
Matrix metalloproteinase 12 (MMP12)	TCAACACATTTCGCCTCTCTGCT	GGATTTGGCAAGCGTTGGTTCT	NM_002426.4
Matrix metalloproteinase-13 (MMP-13)	CCTGATGACGATGTACAAGGGA	TGGCATCAAGGGATAAGGAAGGG	NM_002427.3
Myostatin (MSTN)	TGCTGTAACCTTCCCAGGACCA	GCTCATCACAGTCAAGACCAAAATCC	NM_005259.2
Ribosomal protein, large, P0 (RPLP0)	GGAAACTCTGCATTCTCGCTTCCT	CCAGGACTCGTTTGTACCCGTTG	NM_001002.3
Scleraxis (SCX)	CAGCCCAAACAGATCTGCACCTT	CTGTCTTTCTGTCGCGGTCCTT	NM_001080514.2
Tenascin-C (TNC)	CAACCATCACTGCCAAGTTCACAA	GGGGGTCGCCAGGTAAGGAG	NM_002160.3
Tenomodulin (TNMD)	GAAGCGGAAATGGCACTGATGA	TGAAGACCCACGAAGTAGATGCCA	NM_022144.2
Transforming growth factor-ß1 (TGFB1)	GAGGTCACCCGCGTGCTAATG	CACGGGTTCAGGTACCGCTTCT	NM_000660.5
Transforming growth factor-ß2 (TGFB2)	CCCAAAAGCCAGAGTGCCTGAA	ATGTAGCGCTGGGTTGGAGATG	NM_001135599.2
Transforming growth factor-ß3 (TGFB3)	CTGTGCGTGAGTGGCTGTTGAG	CTCCATTGGGCTGAAAGGTGTG	NM_003239.3
Tumor necrosis factor-α (TNF)	TTCCCCAGGGACCTCTCTCTAATC	GAGGGTTTGCTACAACATGGGCTAC	NM_000594.3
Vascular endothelial GFa (VEGFA)	ATGACGAGGGCCTGGAGTGTGT	CTCCTATGTGCTGGCCTTGGTG	NM_001025366.2

711 Primer sequences used for real-time RT-PCR

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Figure 1

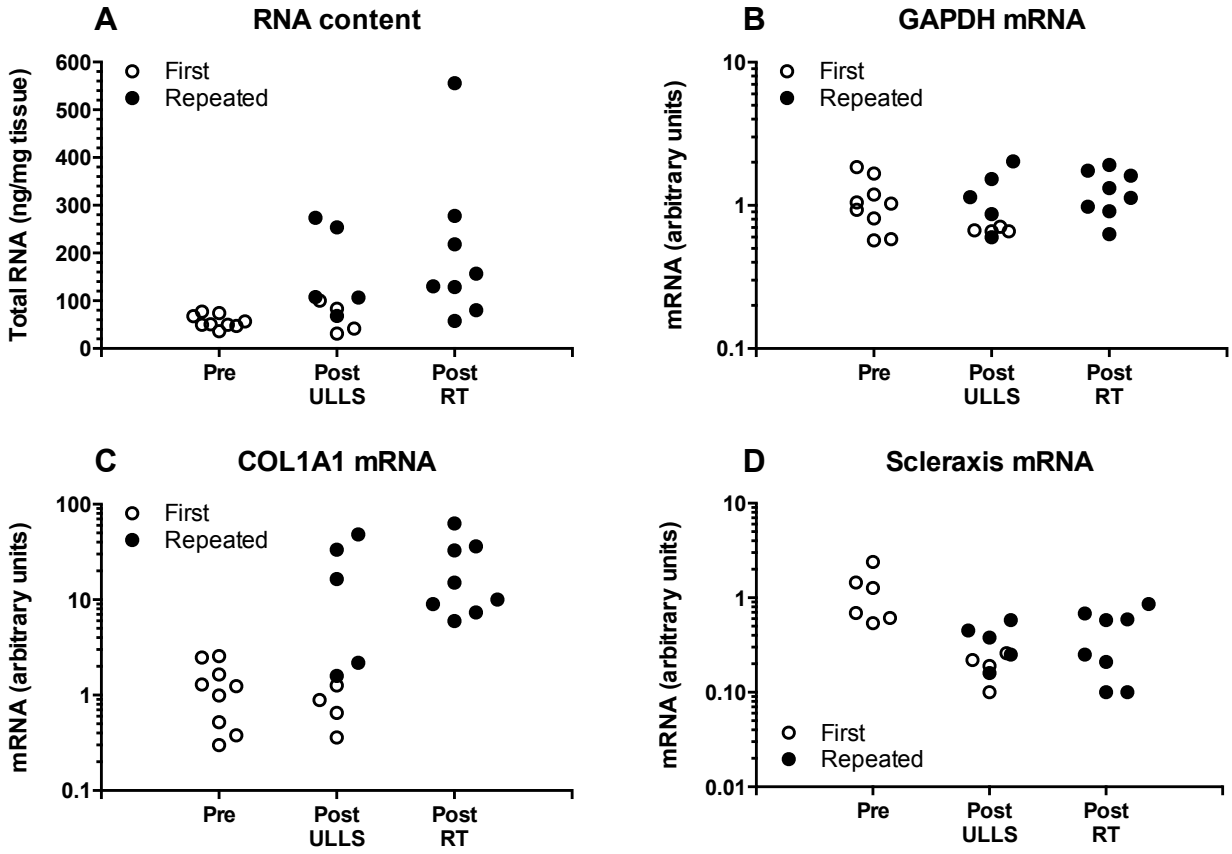

A
RNA content
Total RNA (ng/mg tissue)
600
500
400
300
200
100
0
First
Repeated
Pre
Post ULLS
Post RT
B
GAPDH mRNA
mRNA (arbitrary units)
10
1
0.1
First
Repeated
Pre
Post ULLS
Post RT
C
COL1A1 mRNA
mRNA (arbitrary units)
100
10
1
0.1
First
Repeated
Pre
Post ULLS
Post RT
D
Scleraxis mRNA
mRNA (arbitrary units)
10
1
0.10
0.01
First
Repeated
Pre
Post ULLS
Post RT

Figure 2 – Study II overview

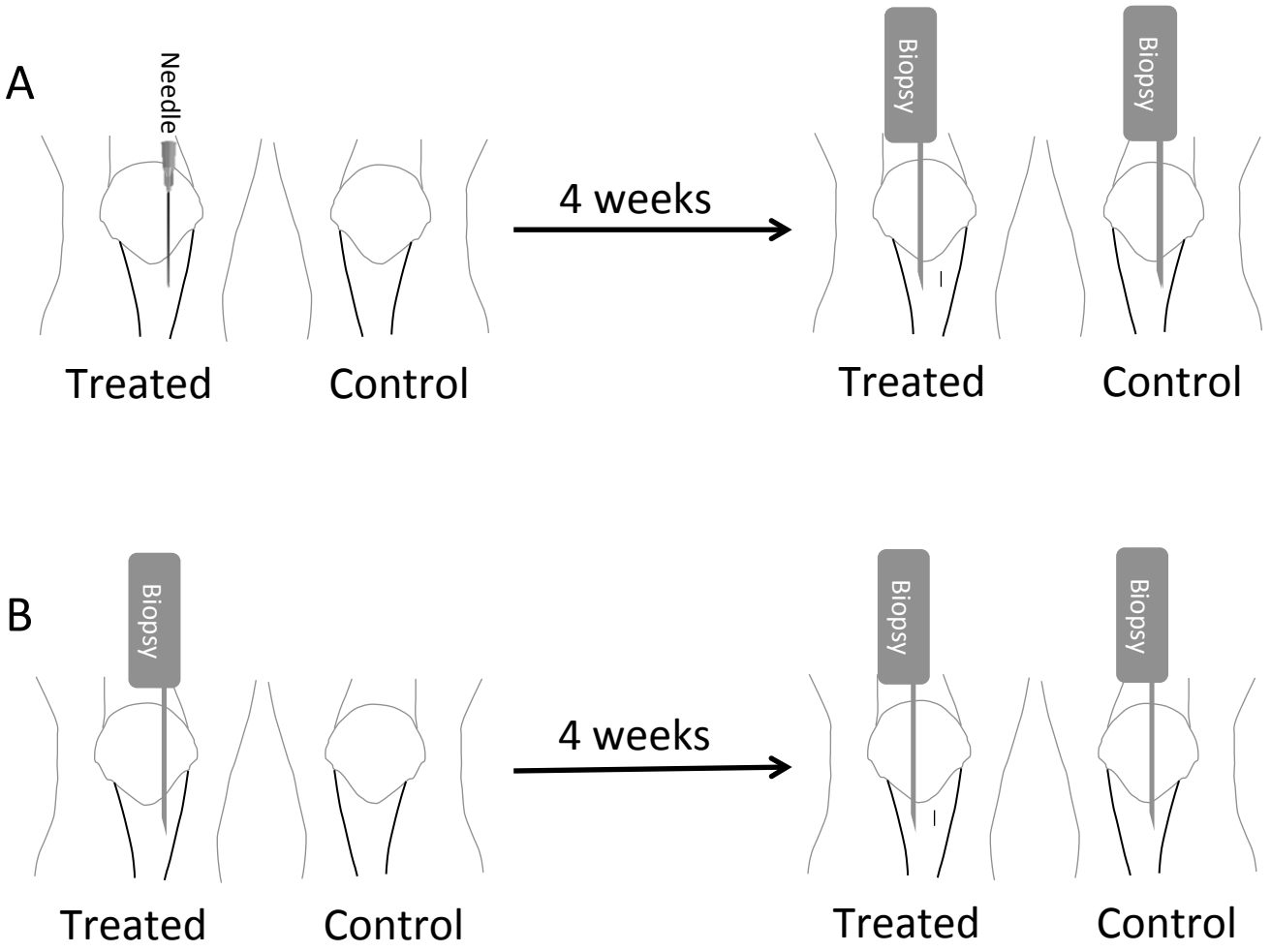


Figure 3

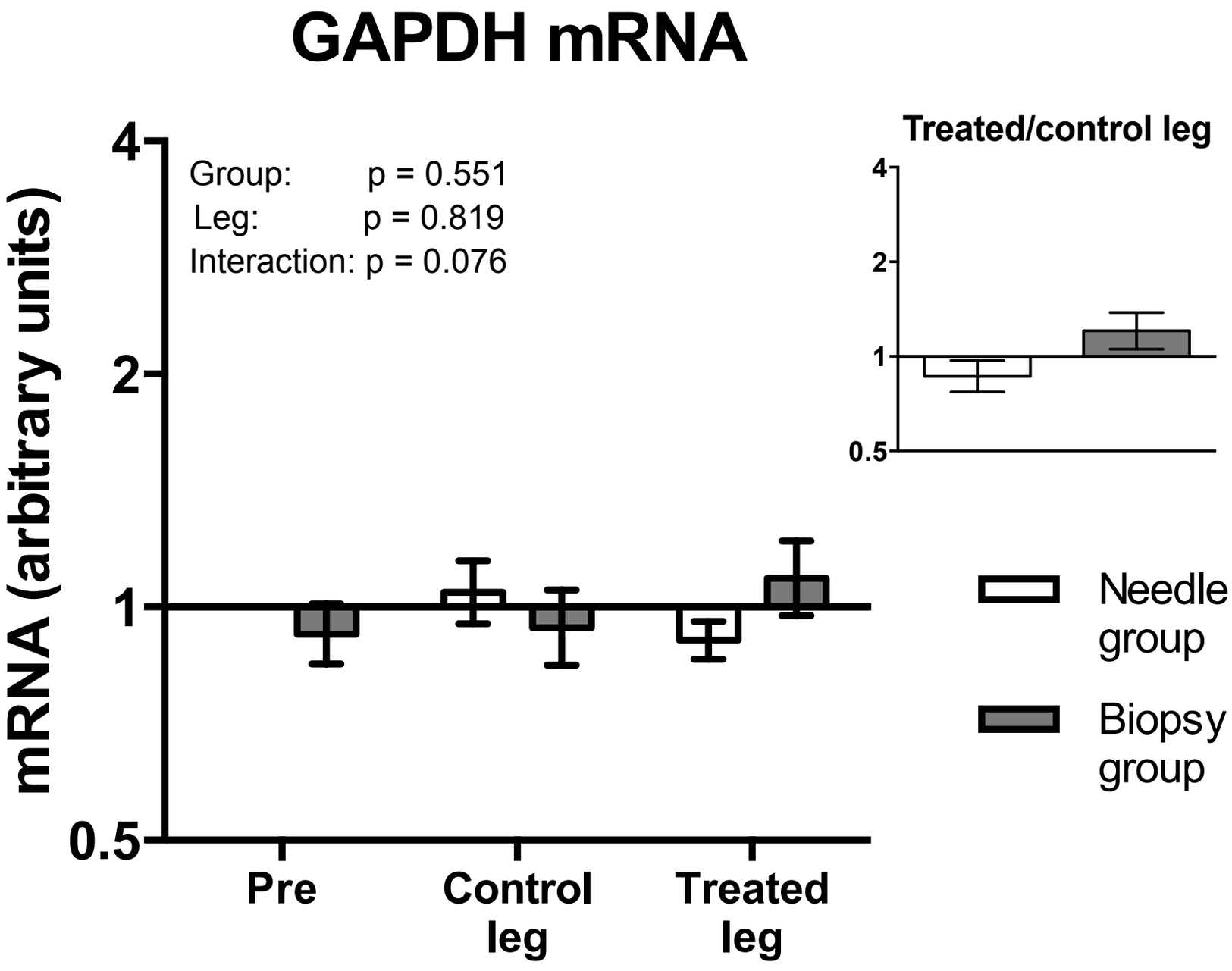

GAPDH mRNA
mRNA (arbitrary units)
4
2
1
0.5
Group: p = 0.551
Leg: p = 0.819
Interaction: p = 0.076
Pre
Control leg
Treated leg
Treated/control leg
4
2
1
0.5
Needle group
Biopsy group

Figure 4

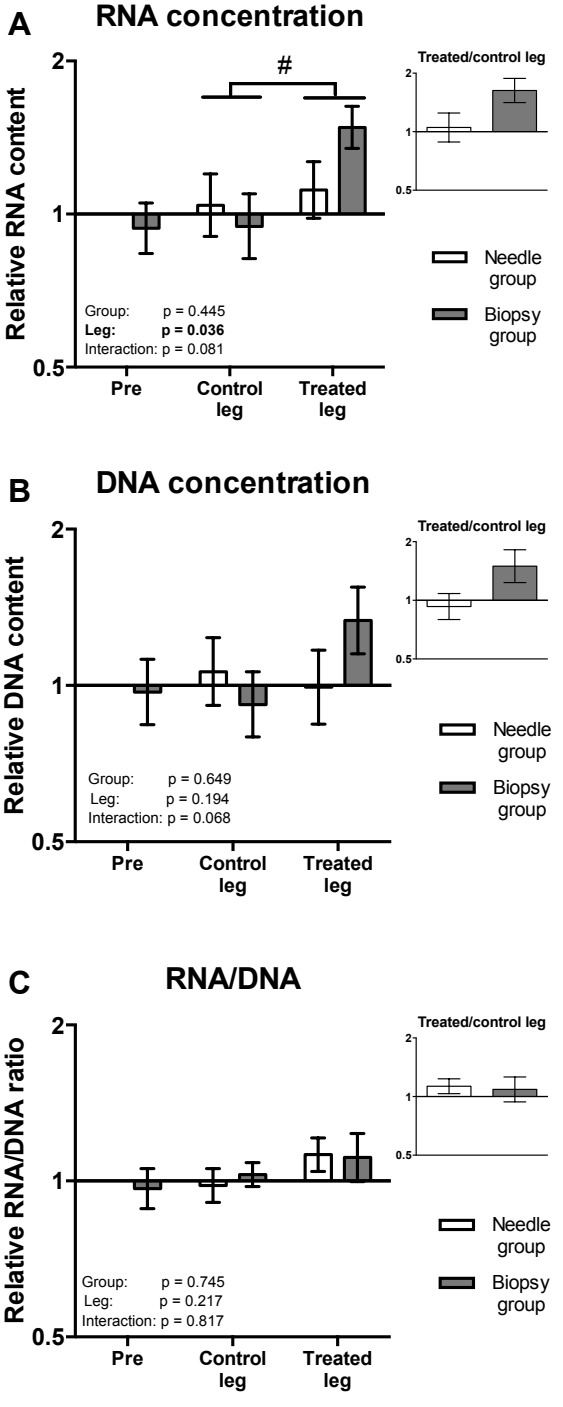

A
RNA concentration
Relative RNA content
2
1
0.5
#
Group: p = 0.445
Leg: p = 0.036
Interaction: p = 0.081
Pre
Control leg
Treated leg
Treated/control leg
2
1
0.5
Needle group
Biopsy group
B
DNA concentration
Relative DNA content
2
1
0.5
Group: p = 0.649
Leg: p = 0.194
Interaction: p = 0.068
Pre
Control leg
Treated leg
Treated/control leg
2
1
0.5
Needle group
Biopsy group
C
RNA/DNA
Relative RNA/DNA ratio
2
1
0.5
Group: p = 0.745
Leg: p = 0.217
Interaction: p = 0.817
Pre
Control leg
Treated leg
Treated/control leg
2
1
0.5
Needle group
Biopsy group

Figure 5

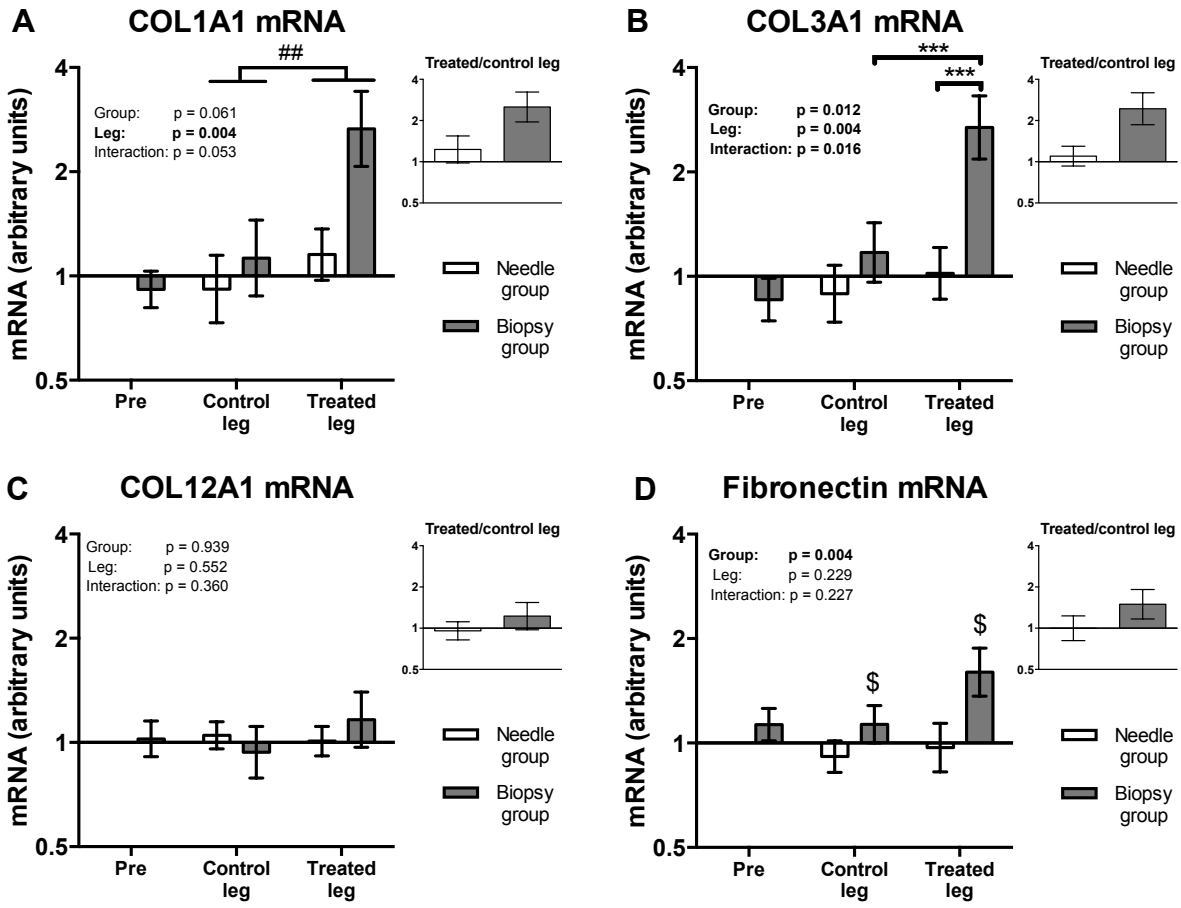
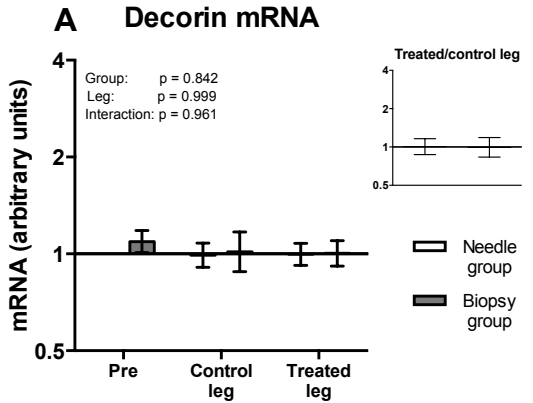
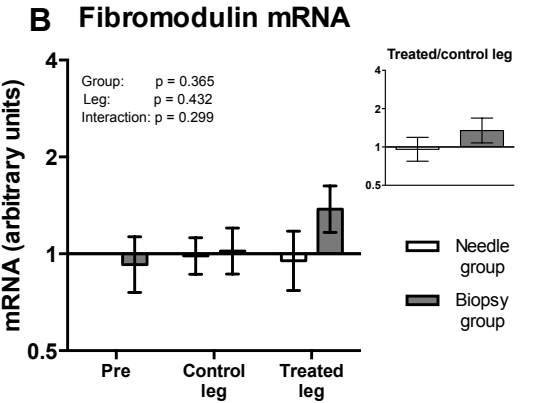
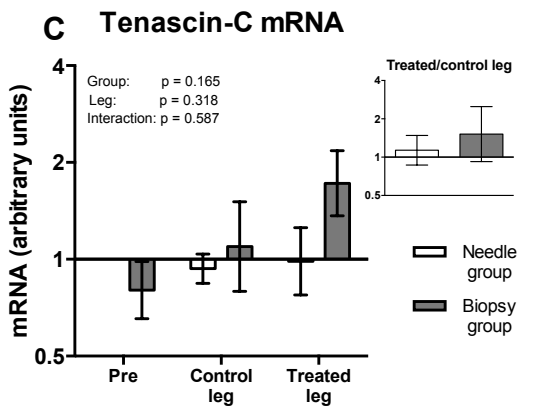

A
COL1A1 mRNA
##
Group: p = 0.061
Leg: p = 0.004
Interaction: p = 0.053
mRNA (arbitrary units)
4
2
1
0.5
Pre
Control leg
Treated leg
Treated/control leg
Needle group
Biopsy group
B
COL3A1 mRNA
***
***
Group: p = 0.012
Leg: p = 0.004
Interaction: p = 0.016
C
COL12A1 mRNA
Group: p = 0.939
Leg: p = 0.552
Interaction: p = 0.360
D
Fibronectin mRNA
Group: p = 0.004
Leg: p = 0.229
Interaction: p = 0.227
$
$

Figure 6


A
Decorin mRNA
Group: p = 0.842
Leg: p = 0.999
Interaction: p = 0.961
mRNA (arbitrary units)
4
2
1
0.5
Pre
Control leg
Treated leg
Treated/control leg
4
2
1
0.5
Needle group
Biopsy group


B
Fibromodulin mRNA
Group: p = 0.365
Leg: p = 0.432
Interaction: p = 0.299
mRNA (arbitrary units)
4
2
1
0.5
Pre
Control leg
Treated leg
Treated/control leg
4
2
1
0.5
Needle group
Biopsy group


C
Tenascin-C mRNA
Group: p = 0.165
Leg: p = 0.318
Interaction: p = 0.587
mRNA (arbitrary units)
4
2
1
0.5
Pre
Control leg
Treated leg
Treated/control leg
4
2
1
0.5
Needle group
Biopsy group

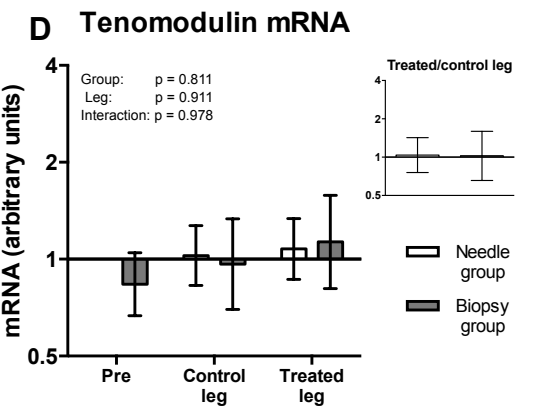

D
Tenomodulin mRNA
Group: p = 0.811
Leg: p = 0.911
Interaction: p = 0.978
mRNA (arbitrary units)
4
2
1
0.5
Pre
Control leg
Treated leg
Treated/control leg
4
2
1
0.5
Needle group
Biopsy group

Figure 7

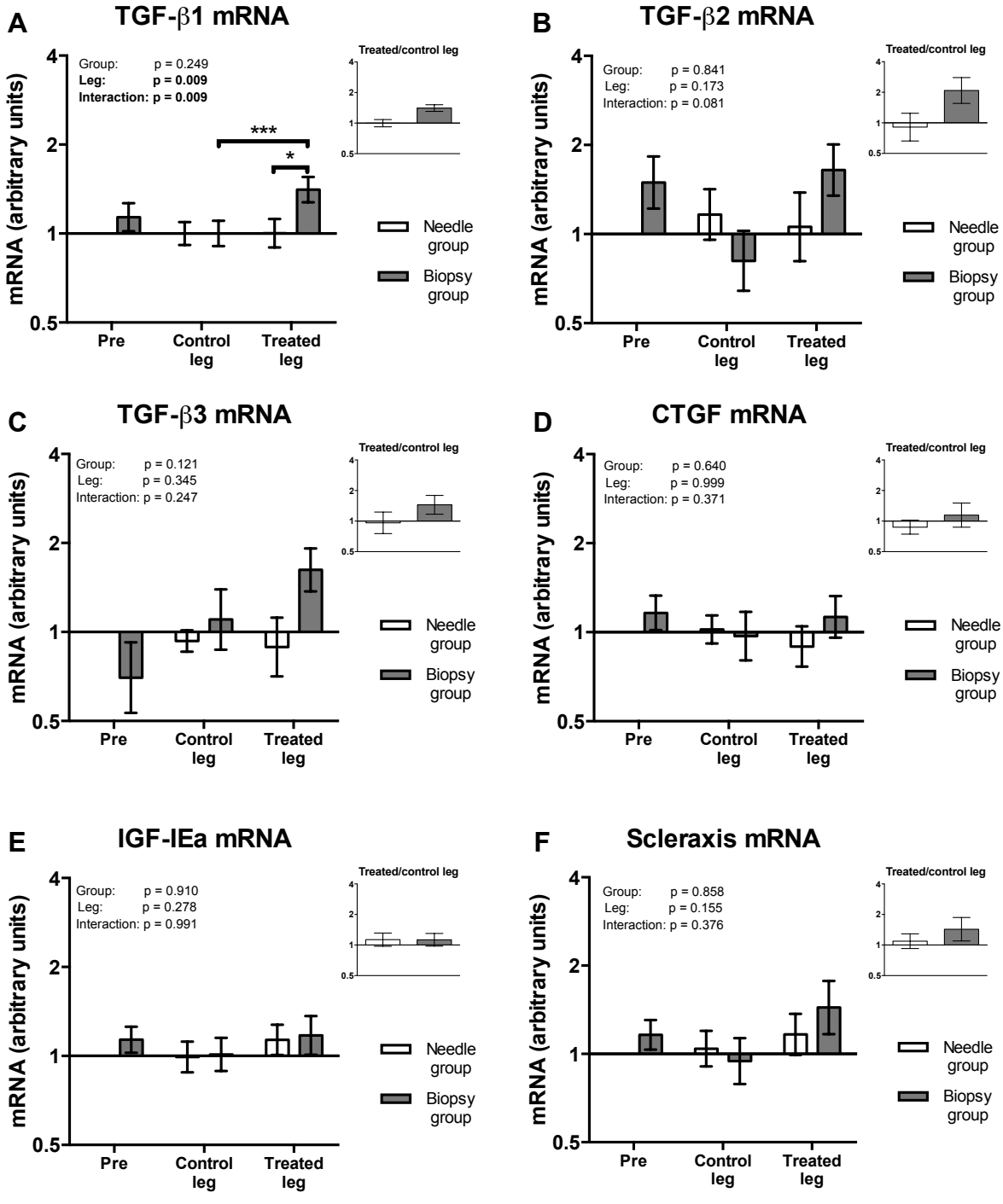
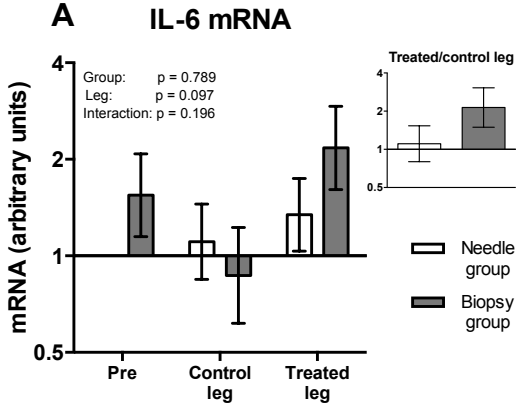
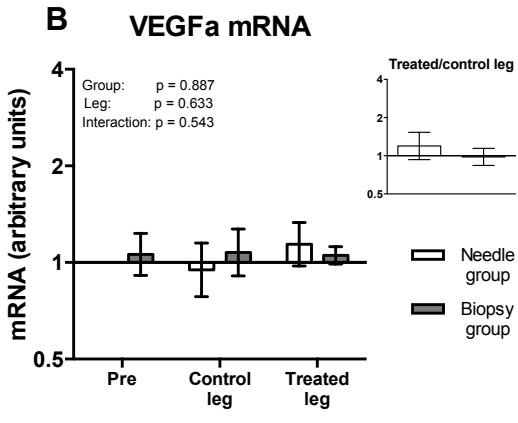
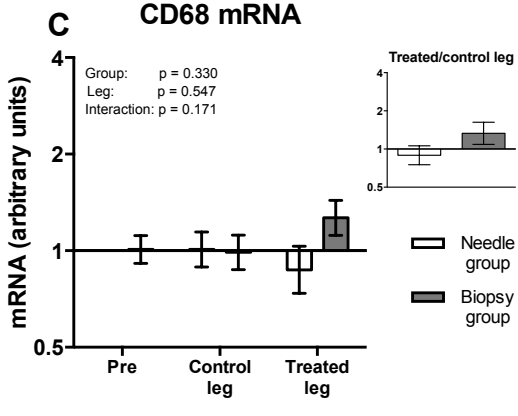

A
TGF-β1 mRNA
Group: p = 0.249
Leg: p = 0.009
Interaction: p = 0.009
***
*
mRNA (arbitrary units)
Pre
Control leg
Treated leg
Treated/control leg
Needle group
Biopsy group
B
TGF-β2 mRNA
Group: p = 0.841
Leg: p = 0.173
Interaction: p = 0.081
C
TGF-β3 mRNA
Group: p = 0.121
Leg: p = 0.345
Interaction: p = 0.247
D
CTGF mRNA
Group: p = 0.640
Leg: p = 0.999
Interaction: p = 0.371
E
IGF-IEa mRNA
Group: p = 0.910
Leg: p = 0.278
Interaction: p = 0.991
F
Scleraxis mRNA
Group: p = 0.858
Leg: p = 0.155
Interaction: p = 0.376

Figure 8


A
IL-6 mRNA
Group: p = 0.789
Leg: p = 0.097
Interaction: p = 0.196
mRNA (arbitrary units)
4
2
1
0.5
Pre
Control leg
Treated leg
Treated/control leg
4
2
1
0.5
Needle group
Biopsy group


B
VEGFa mRNA
Group: p = 0.887
Leg: p = 0.633
Interaction: p = 0.543
mRNA (arbitrary units)
4
2
1
0.5
Pre
Control leg
Treated leg
Treated/control leg
4
2
1
0.5
Needle group
Biopsy group


C
CD68 mRNA
Group: p = 0.330
Leg: p = 0.547
Interaction: p = 0.171
mRNA (arbitrary units)
4
2
1
0.5
Pre
Control leg
Treated leg
Treated/control leg
4
2
1
0.5
Needle group
Biopsy group