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Minocycline microspheres did not significantly improve outcomes after collagenase injection of tendon

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A B S T R A C T

Background: Tetracycline antibiotics inhibit matrix metalloproteinases and pro-inflammatory cytokines implicated in the pathogenesis of tendinopathy, while microsphere formulations allow sustained release of drug contents. The purpose of this study was to evaluate the ability of a local minocycline microsphere injection to restore normal tendon properties in a rat model of collagenase-induced patellar tendinopathy.
Methods: A total of 22 rats were randomly assigned to the control (n = 11) or minocycline (n = 11) group and received bilateral patellar tendon injections of collagenase. After 7 days, the minocycline group received the minocycline microsphere treatment and the control group received phosphate buffered solution. Pain was assessed via activity monitors and Von Frey filament testing. At 4 weeks post-collagenase injections, animals were euthanized.
Results: Cage crossings significantly decreased among all rats 2–3 days following each injection period, however, tactile allodynia measures did not reflect this injury response. Biomechanical properties, interleukin-1 beta levels, and glycosaminoglycan content did not differ between groups. While not statistically significant, levels of leukotriene B_4 were lower in the minocycline group compared to controls ($p = 0.061$), suggesting a trend.
Conclusions: Our study further characterizes the collagenase model of tendinopathy by demonstrating no evidence of central sensitization with collagenase-induced injury. We found no adverse effect of intratendinous injections of minocycline-loaded poly-lactic-co-glycolic acid microspheres, although no therapeutic effect was observed. Future studies involving a more substantial tendon injury with a greater inflammatory component may be necessary to more thoroughly evaluate the effects of minocycline on tendon pathology.

1. Introduction

Tendinopathy is a debilitating and prevalent disorder that remains difficult to treat. The pathologic tendon has degeneration and disorganization of the collagen matrix, increased vascularization, and increased cellularity. Tendinopathy can be manifested as pain, tendon thickening, and diminished biomechanical properties increasing the risk of tendon rupture.[1]

Although the exact etiology is unknown, proposed mechanisms of tissue damage in tendinopathy include hypoxia, ischemia, oxidative stress, induced apoptosis, and the production of inflammatory cytokines.[2] Matrix metalloproteinases (MMPs) have been characterized as the mediators of collagen degradation in tendons, and are thus implicated in the pathogenesis of tendinopathy.[3] The balance between collagen breakdown and synthesis is partially maintained by endogenous tissue inhibitors of matrix metalloproteinases (TIMPs). In the pathologic tendon, however, MMP activity increases and TIMP activity decreases, resulting in net tissue degeneration.[4] The remodeled extracellular matrix is characterized by increased water content and levels of sulfated glycosaminoglycans (GAG) compared to healthy tendons.[5]

The development of tendinopathy may in part be regulated by pro-inflammatory cytokines. Rat tendons subjected to repetitive overuse injuries contain higher levels of IL-1β, a cytokine that has been shown to induce MMP secretions from human tendon cells, induce inflammatory mediators, and suppress type I collagen.[6,7] Both sides of the arachidonic acid breakdown pathway have been implicated in tendinopathy as cyclic stretching of tendon fibroblasts is associated with increased levels of both prostaglandin E_2 (PGE_2) and leukotriene B_4 (LTB_4). Inhibition of cyclooxygenase (COX) enzymes via non-steroidal anti-inflammatory medications (NSAIDs) has been found to increase LTB_4 levels, likely as a result of a shunting effect within the arachidonic acid pathway.[8] It has been observed that the 5-lipoxygenase (5-LO) pathway may be more active than the COX pathway following tendon overuse, suggesting a therapeutic role of 5-LO inhibition.[9]

Tetracycline antibiotics have been found to exhibit anti-inflammatory and anti-nociceptive properties in addition to their antimicrobial effects. The ability of tetracyclines to inhibit MMPs in the setting of tissue injury is well characterized in the literature.[3,10] While

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the optimal timing of delivery remains unclear, extended delivery of tetracyclines beyond 2 weeks is associated with the amelioration of fibrosis development and improved biomechanical properties of rat tendons following transection and repair.[10] Minocycline has additionally been found to suppress 5-LO expression, thus reducing levels of pro-inflammatory leukotrienes.[11,12] This property likely contributes to the decreased rat paw edema and peritoneal leukocyte migration observed following delivery of an inflammatory stimulus.[13] The anti-nociceptive effects of tetracyclines are evidenced by the inhibition of carrageenan-induced mechanical allodynia 1 h after doxycycline or minocycline administration.[14] As stress deprived tendons have been noted to exhibit increased MMP activity, pain control may play a critical role in preventing limb disuse and resultant tendon degeneration.[15] The attenuation of the acute inflammatory response and the anti-nociceptive effects of tetracyclines therefore contribute to the promising therapeutic potential of these antibiotics in the setting of tendinopathy.

Arestin is an FDA-approved minocycline microsphere formulation currently used in the treatment of periodontitis. Locally delivered minocycline microspheres have been found to significantly reduce IL-1 levels and inhibit tissue destruction clinically in periodontal disease.[16] Systemic drug administration is undesirable due to difficult drug dosing and potentially unwanted side-effects. The goal of local delivery of biodegradable microspheres is to maintain high drug concentration at the site of injury for prolonged duration. The release pattern of tetracycline-loaded poly-lactic-co-glycolic acid (PLGA) microspheres is associated with minimal cytotoxicity, as evidenced by low lactate dehydrogenase activity, and a steady release of the drug over 20 days.[17] The purpose of this study was to evaluate the ability of a local minocycline microsphere injection to restore normal tendon properties in a rat model of collagenase-induced patellar tendinopathy. We hypothesized that tendons treated with minocycline microspheres 7 days post-injury would demonstrate improved biomechanical properties, decreased GAG content, decreased levels of pro-inflammatory cytokines, and lower histopathology grades at 28 days post-injury when compared to tendons injected with PBS.

2. Materials and methods

Protocols were approved by the University Institutional Animal Care and Use Committee. A total of 22 female Sprague-Dawley retired-breeder rats (mean age = 24 weeks) were assigned randomly into one of two groups: a control group (CON) receiving collagenase injection followed by PBS injection (n = 11) and an intervention group receiving collagenase injection followed by minocycline microsphere (MIN) injection (n = 11). Collagenase injection was chosen as the resulting injury exhibits many features consistent with tendinopathy such as hypercellularity, loss of matrix organization, increased vascularity, and failed healing.[18] Collagenase was prepared by suspending 100 mg crude type I collagenase in 10 mL PBS. The resultant mixture was centrifuged for 10 min at 1,000 g to reveal a pellet of debris. The supernatant was extracted and filtered through a sterile nylon syringe filter. The sterilized collagenase mixture was divided into 1 mL aliquots and stored at −20 °C.

Beginning three days prior to the collagenase injections and continuing to termination, rats were provided with 1.6 mg/mL acetaminophen (200 mg/kg) in their drinking water for analgesia. Animals were anesthetized with isoflurane prior to injection. All injections were performed bilaterally with the knee flexed to 90° to tension the tendon. In a similar method to previous studies at this institution, a 27-guage needle was inserted midway between the origin and insertion of the patellar tendon, angled towards the distal pole of the patella at the insertion of the patellar tendon.[19] At the start of the study, both CON and MIN groups received 25 µl of 10 mg/mL Type IA collagenase. Seven days following collagenase injection, the CON group received 50 µl of PBS, while the MIN group received 50 µl of the treatment solution containing 0.25 mg/mL minocycline microspheres. To prepare the minocycline treatment, 1 mg of dry microspheres was reconstituted in 4 ml diluent containing sterile aqueous solution of PBS, sodium carboxymethylcellulose (NaCMC; 0.5% w/w), and polysorbate-80 (0.1% w/w).[20] At the time of each procedure, rats were given a subcutaneous injection of 0.03 mg/kg buprenorphine for pain control. At 28 days post-injury, the animals were euthanized by way of carbon dioxide overdose followed by thoracotomy. A lateral limb x-ray was then taken for each rat to evaluate tendon length.

2.1. Pain assessments

For a subset of 6 rats per group, the number of cage crossings per day were measured using a photoelectric sensor system. Activity measurements began 3 days prior to collagenase injections to establish baseline activity levels and continued until euthanasia. For the other 5 rats per group, tactile allodynia was estimated using Von Frey filament testing to determine 50% response thresholds, where lower threshold corresponds to increased sensitivity.[21] Tactile allodynia was assessed at baseline, 2 days after each injection procedure period, and 4 days prior to euthanasia.

2.2. Biomechanical testing

All of the right patellar tendons (n = 11/group) were used for biomechanical testing. The cross-sectional area of each tendon was measured prior to failure testing. A materials testing system (Instron 8500 Plus, Instron Corporation, Norwood, MA) tensioned the tendons to failure, while plotting a load-displacement curve. Ultimate load and stiffness values were extracted from this curve, while material properties were computed thereafter (i.e. elastic modulus = stiffness x length/area, tensile strength = ultimate load/area).

2.3. Histology evaluation

Five left patellar tendons per group were prepared using previously described methods for tendon histology.[22] Following preparation, slides were scanned with the Aperio ScanScope XT slide scanning system, and viewed using Imagescope viewing software (Aperio Technologies, Vista, CA). Twelve regions of interest (ROI) were identified per tendon and graded by 3 blinded observers. ROIs were each 500 × 400µm unique regions of tendon. A previously described grading system was used to evaluate fiber arrangement, nuclear rounding, angiogenesis, and cell density.[23] For each variable, ROIs were graded from 0 to 3, with 0 representing normal tendon histology and 3 representing maximum pathology. Thus, a normal tendon would score 0 and a maximally abnormal tendon would score 12. Scores were then averaged across ROIs and observers to obtain an average score for each tendon.

2.4. GAG evaluation

All of the right patellar tendons (N = 11/group) were harvested and weighed immediately after biomechanical testing, then stored at −80 °C until the time of use. Tendons were thawed and digested in papain for 24-h at 65 °C. The dimethylmethylene blue assay was used to quantify tendon glycosaminoglycan (GAG) content.[24]

2.5. IL-1β, and LTB_4 assays

Six of the left patellar tendons per group were harvested at the time of euthanasia and stored at −80 °C until the time of use. Tendons were homogenized using a cryogrinder and suspended in 0.5 ml of 95% ethanol. After centrifugation, the supernatant was transferred to a new tube and evaporated, leaving behind the fatty acid fraction for LTB_4 evaluation (LTB_4 Assay, R&D Systems, Minneapolis, MN). The remaining tissue was re-suspended in 0.5 ml Mammalian Protein Extraction Reagent (MPER) and placed on a shaker for 30 min. After centrifuging again, the supernatant was transferred to a new tube for IL-

1β evaluation (ER2IL1B Pierce Biotechnology, Rockford, IL). All results were normalized to tendon weight.

2.6. Statistical analysis

Activity monitor and Von Frey filament data were evaluated using two-way repeated measures ANOVA to detect statistical differences across time points and between groups. Independent T-tests were used to compare all other variables between groups. Statistical significance was established a priori at $\alpha \leq 0.05$.

3. Results

Three days after collagenase injections, the mean number of cage crossings among all rats significantly decreased from an average daily baseline of 725 ± 152 to 546 ± 104 crossings ($p = 0.008$). Cage crossings then increased above baseline on post-injury day 6–920 ± 304 daily crossings ($p = 0.004$). On post-injury day 9, two days after minocycline or PBS injections, average activity significantly decreased from pre-treatment levels to 422 ± 183 daily crossings ($p = 0.001$). Both groups returned to baseline activity by post-injury day 13. There were no significant activity differences between groups at any time point (Fig. 1).

The results of the Von Frey filament testing are outlined in Fig. 2. There were no significant differences between groups in regard to 50% response thresholds at any time point. The average 50% response thresholds combined for both groups were 19.3 ± 3.7g, 20.5 ± 2.9g, 19.8 ± 3.4g, and 19.7 ± 2.9 g at baseline, 2 days post-collagenase, 2 days post-treatment, and 4 days prior to euthanasia, respectively.

There were no significant differences between groups in regard to tendon length (CON = 8.74 ± 0.20 mm, MIN = 8.82 ± 0.30 mm) or cross-sectional area (CON = 5.37 ± 1.40 mm^2, MIN = 5.45 ± 0.62 mm^2). Mean load at tendon failure (CON = 76.0 ± 10.4 N, MIN = 75.9 ± 19.6 N), stiffness (CON = 68.0 ± 13.8 N/mm, MIN = 62.2 ± 14.4 N/mm), tensile strength (CON = 14.9 ± 4.0 N/mm^2, MIN = 14.0 ± 3.9 N/mm^2), and elastic modulus (CON = 115.9 ± 31.9 N/mm^2, MIN = 102.3 ± 29.2 N/mm^2) were similar between groups (Table 1).

One tendon from the CON group and two tendons from the MIN group were excluded from LTB_4 calculations because of contamination during preparation. Average LTB_4 levels for the control tendons were 5.98 ± 2.04 ng/mL (n = 5) compared to minocycline-treated tendons at 3.28 ± 1.44 ng/mL (n = 4), although this decrease was not significant ($p = 0.061$; Fig. 3). The IL-1β levels (CON = 2.06 ± 0.21 ng/mL, MIN = 1.84 ± 0.46 ng/mL; $p = 0.32$) and GAG content (CON = 0.31 ± 0.12 mg/mL, MIN = 0.28 ± 0.13 mg/mL; $p = 0.615$) did not exhibit statistically significant differences between groups.

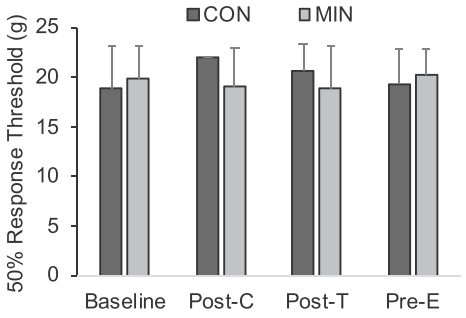

Fig. 2. Fifty percent response thresholds from Von Frey filament testing. There were no threshold differences between groups or time points (Post-Collagenase = 2 days following injection, Post-Treatment = 2 days following injection, Pre-Euthanasia = 4 days prior to euthanasia).

Table 1
Biomechanical properties of patellar tendons. There were no significant differences in biomechanical properties between groups (mean ± SD).

	CON	*MIN*	*p value*
Cross-sectional Area (mm^2)	5.4 ± 1.4	5.5 ± 0.6	0.857
Load at Tendon Failure (N)	76.0 ± 10.4	75.9 ± 19.6	0.986
Stiffness (N/mm)	68.0 ± 13.8	62.2 ± 14.4	0.352
Tensile Strength (N/mm^2)	14.9 ± 4.0	14.0 ± 3.9	0.607
Elastic Modulus (N/mm^2)	115.9 ± 31.9	102.3 ± 29.2	0.322

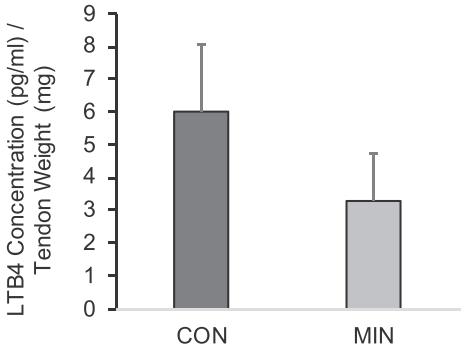

Fig. 3. Leukotriene B_4 levels in rat patellar tendon tissue. Tendons treated with minocycline had lower levels of leukotriene B_4 (LTB_4), but the difference was not significant (p = 0.061).

Table 2 depicts the histopathology scores for both groups and Fig. 4 illustrates representative sections for the CON and MIN tendons. Total pathology scores were similar between the CON and MIN groups

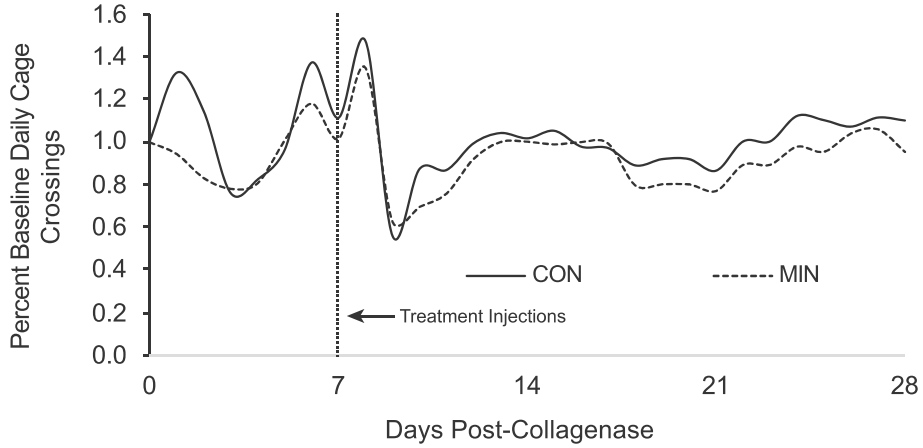

Fig. 1. Average number of rat daily cage crossings. The number of daily cage crossings significantly decreased 2–3 days after each injection time point. There was no difference between groups in rate of recovery following decreases in activity.

Table 2
Histopathology scores of patellar tendon sections. Values represent the mean scores of 3 graders across 12 regions of interest per tendon. There were no significant differences in histopathology scores between groups (mean ± SD).

	CON	MIN	p value
Fiber Arrangement	1.07 ± 0.17	1.12 ± 0.20	0.682
Angiogenesis	0.38 ± 0.27	0.70 ± 0.16	0.051
Nuclear Rounding	1.28 ± 0.22	1.17 ± 0.07	0.322
Cell Density	1.17 ± 0.58	1.68 ± 0.10	0.084
Total Pathology Score	3.89 ± 0.98	4.67 ± 0.43	0.144

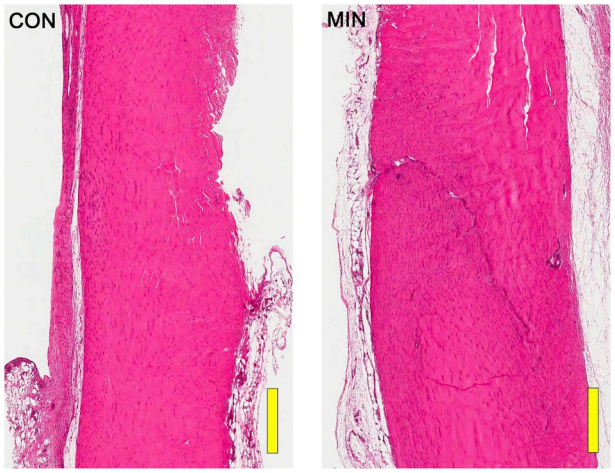

Fig. 4. Representative histology sections of rat patellar tendons collected 28 days post-injury and evaluated with hematoxylin-eosin (HE) staining. Minocycline did not significantly improve the histologic appearance of tendons following injury. Magnification = 4x, scale bars = 400 μm.

(CON = 3.89 ± 0.98, MIN = 4.67 ± 0.43). There were no significant differences in average pathology scores for fiber arrangement, angiogenesis, nuclear rounding, or cell density. However, the minocycline treated tendons displayed greater angiogenesis (CON = 0.38 ± 0.27, MIN = 0.70 ± 0.16; $p = 0.051$) and cell density (CON = 1.17 ± 0.58, MIN = 1.68 ± 0.10; $p = 0.084$) compared to controls, although neither of these increases was statistically significant.

4. Discussion

This study demonstrated that minocycline microsphere injections did not significantly improve outcomes in a collagenase-induced model of patellar tendinopathy. We hypothesized that minocycline microsphere injection would reduce inflammation and degenerative changes of the patellar tendon following collagenase injury. However, the biomechanical properties, inflammatory markers, and histological findings were similar between PBS and minocycline treated tendons. These findings differ from prior studies characterizing the favorable effects of tetracycline antibiotics during tendon healing after acute transection injury.[10,25] However, this is the first study investigating the effects of local administration of minocycline in a collagenase-induced tendinopathy model. Prior studies have utilized oral doxycycline, noting therapeutic effects following transection and repair.[10,25] The injury induced by transection and repair differs from that of collagenase, and these results may not be directly comparable. Reduction of MMP activity to basal levels has been reported to have a positive effect in the setting of tendinopathy.[4] Doxycycline is a more potent inhibitor of MMPs than minocycline, potentially contributing to this discrepancy of findings.[3] The delayed timing of minocycline delivery in the present study may further contribute to the lack of observed effect. In the present study, a one-week recovery period between injections was required by the animal care committee to allow for diminished swelling. MMP inhibition during a time of peak inflammation shortly following the collagenase injury may lead to a more therapeutic response.

Although not statistically significant, levels of LTB_4 were lower in the minocycline group compared to controls, suggesting a trend. Prior studies have shown that minocycline inhibits the activity of 5-LO in neuronal cell lines and in CNS tissue following ischemic injury.[11,12] Within the CNS, the mechanism by which minocycline exerts its anti-inflammatory effects may be related to both the downregulation of 5-LO expression and the inhibition of 5-LO enzymatic activity.[11] However, the 5-LO inhibitory effect of minocycline has not been well characterized in tendon, and the magnitude of 5-LO inhibition in the setting of tendinopathy may differ from its inhibitory effects following CNS ischemic injury. The small sample size used for calculating LTB_4 levels may additionally contribute to the lack of significance.

While there is evidence to support minocycline's ability to inhibit multiple pathways currently implicated in the development tendinopathy, we did not identify a positive response in the tendons treated with minocycline. Minocycline has been shown to inhibit IL-1β, but no differences were detected between groups at 4-weeks post-injury. In a study of tendon overuse by Gao et al., IL-1β was significantly increased immediately after a high-repetition, low-force training period. However, the increased levels of IL-1β quickly returned to baseline during a period of rest.[6] In the present study, IL-1β may have transiently increased following injury with resolution to baseline prior to 4-weeks, potentially accounting for the lack of difference between groups. Additionally, biomechanical properties and histopathology scores were not improved in minocycline-treated tendons. Although not statistically significant, histopathology scores were slightly higher in the MIN group, a finding potentially attributable to increased angiogenesis and cell density. This may in part be a result of a shunting effect within the arachidonic acid pathway, with inhibition of 5-LO causing an increase in prostaglandin production. It has previously been noted that both PGE_2 and LTB_4 levels increase following cyclic stretching of tendon fibroblasts, and that blocking prostaglandin production leads to increased leukotriene production and vice versa.[8] If minocycline inhibited 5-LO, this may have resulted in shunting of the arachidonic acid breakdown pathway toward increased prostaglandin production. This effect was an anticipated possibility, but it is unclear if prostaglandins have a net positive or negative effect on the remodeling of damaged tendons. Prior studies in our lab have demonstrated improved tendon strength and stiffness properties following weekly peripatellar injections of PGE_2.[19] However, repeated exposure of Achilles tendons to PGE_1 has been associated with hypercellularity, proliferation of capillaries, and tendon degeneration.[26] Thus, the findings observed in MIN group histologic sections may, in part, be the result of increased prostaglandin production, but these effects may be important for structural remodeling of tendons. In a rotator cuff transection and repair model, Oak et al. observed that oral administration of a dual inhibitor of both 5-LO and COX enzymes reduced inflammation and improved biomechanical properties of healing tendons.[27] Additional studies involving the inhibition of both the 5-LO and COX pathways may be useful in determining the role of prostaglandins following tendon injury.

Based on Von Frey filament testing, there was no evidence of central sensitization in the present study. Despite decreases in cage crossings over time, 50% response thresholds were not significantly different at any time point. This finding is consistent with prior studies characterizing tendinopathy as a peripheral pain state lacking features of central sensitization.[28] However, the observed pain response may have been partially diminished as animals received acetaminophen in their drinking water throughout the study, in accordance with animal care requests. Minocycline did not seem to influence pain in the present study, as rats receiving minocycline injections did not show differences in ambulatory activity or 50% response thresholds compared to rats receiving PBS injections. Minocycline has been effective in reducing paw pain following carrageenan and formalin injection, but this effect may be specific to these models.[13,14] The injury caused by carrageenan and formalin may have a greater inflammatory component than collagenase, potentiating a greater nociceptive effect with minocycline.

One potential limitation of the present study is the degree of tendon pathology. While histological sections confirmed some level of tendon damage in both groups, our histopathology scores were much lower when compared to prior studies of tendinopathy. Chen et al. utilized the same tendon histology grading system as the present study and noted greater histopathology scores in a collagenase-induced model of Achilles tendinopathy. At 4 weeks post-collagenase injection, observers reported average pathology scores > 2 for fiber arrangement, angiogenesis, and nuclear rounding, indicating moderate to severe abnormalities.[29] In the present study, average pathology scores were < 2 for fiber arrangement, nuclear rounding, and cell density, with nearly normal scores for angiogenesis, indicating less severe pathology. Our pathology scores for fiber arrangement, angiogenesis, nuclear rounding, and cell density were 41%, 32%, 54%, and 44% of the scores reported by Berkoff et al. in a carrageenan-induced model of tendinopathy.[30] Tendon cross-sectional areas in the present study were significantly smaller than those observed by Berkoff et al., further suggesting limited injury.[30] The low levels of tissue injury may have led to less impactful changes in biomechanical properties and GAG content. Alternatively, there is evidence to suggest that tetracycline antibiotics may not influence tendon swelling. Kessler et al. found that doxycycline administration improved most biomechanical properties, but did not significantly reduce cross-sectional area of Achilles tendons following transection and repair.[10] Nonetheless, future studies involving a more severe tendon injury with a greater inflammatory component may be necessary to better characterize the ability of minocycline to restore normal tendon properties to damaged tendons.

This study demonstrated no significant changes in tendon structure or histology following treatment with minocycline microspheres in a collagenase-induced model of tendinopathy. The study did, however, illustrate the potential viability of the treatment model. Based on our observations, there was no adverse response to intratendinous injections of minocycline-loaded PLGA microspheres. Additionally, this study further characterized the collagenase model of tendinopathy by demonstrating no evidence of central sensitization with collagenase-induced injury.

Despite promising evidence that minocycline may reduce acute inflammation and degenerative changes in the setting of tendinopathy, we were unable to detect a therapeutic effect. Future studies involving larger samples and a more significant or more chronic tendon injury with a greater inflammatory component may be necessary to more thoroughly evaluate the effects of minocycline on tendon pathology. The development of effective treatments of tendinopathy will be aided by the continued investigation of the pathogenesis of the condition. Further exploration of the mediators of collagen degradation and their chronology will guide future studies of novel microsphere preparations as well as the optimal timing for delivery of drug-loaded microspheres in the setting of tendinopathy.

Conflicts of interest

None.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jor.2019.06.007.

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