Enhancement of Achilles Tendon Repair Mediated by Matrix Metalloproteinase Inhibition via Systemic Administration of Doxycycline

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ABSTRACT: Collagenases or matrix metalloproteinases (MMPs) have been shown to play an important role in the matrix degradation cascade associated with Achilles tendon rupture and disease. The goal of this study was to examine the effects of daily administration of doxycycline (Doxy) through oral gavage on MMP activity and on the repair quality of Achilles tendons in vivo. Our findings indicate that Achilles tendon transection resulted in increasing MMP-8 activity from 2 to 6 weeks post-injury, with peak increases in activity occurring at 4 weeks post-injury. Doxy adiministration at clinically relevant serum concentrations was found to significantly inhibit MMP activity after continuous treatment for 4 weeks, but not for continuous administration for shorter durations (96 h or 2 weeks). Extended doxy administration was also associated with improved collagen fibril organization, and enhanced biomechanical properties (stiffness, ultimate tensile strength, maximum load to failure, and elastic toughness). Our findings indicate that a temporal delay exists between Achilles tendon transection and associated increases in MMP-8 activity in situ. Our findings suggest that inhibition of MMP-8 at its peak activity levels ameliorates fibrosis development and improves biomechanical properties of the Achilles tendon. © 2013 Orthopaedic Research Society. Published by Wiley Periodicals, Inc. J Orthop Res 32:500–506, 2014.

Keywords: achilles tendon; matrix metalloproteinase; doxycycline; tendon rupture and repair

Achilles tendon tears are devastating injuries, and have unpredictable outcomes with respect to return to function.[1,2] Collagenases or matrix metalloproteinases (MMPs) have been shown to play an important role in the enzymatic matrix degradation cascade associated with tendon injury and disease.[3–6] Tendon degeneration is an active, cell-mediated process that may result from a failure to regulate specific MMP activities in response to repeated injury or mechanical strain. After tendon rupture, increased activity of MMPs and increased matrix turnover have been associated with further deterioration in the quality of the collagen network.[7–13] Downregulation of tissue inhibitors of MMPs (TIMPS) is also associated with disregulation of the ratio of MMP to TIMP activity, and has been implicated in the pathogenesis of tendinopathy.[8–16] Tenocyte production of MMP-1 and -3 underscores the potential for non-lymphocyte mediated cytokine production of proteases inducing local matrix changes.[17]

Tetracyclines, through a mechanism independent of their antibiotic effects, have been shown to substantially inhibit the action of MMPs, such as collagenase.[18–21] Specifically, doxycycline (doxy) is considered to be the most potent tetracycline MMP inhibitor, whose activity is mediated both by direct and indirect mechanisms of action, and has been applied therapeutically in a variety of clinical applications.[18–21] The use of doxy in tendon repair had been previously explored in vivo, though contradictory findings regarding its efficacy have been reported.[22–24] Consequently, MMP inhibitors and their activity may have very different effects based on timing of administration and type of repair model examined.

The goal of this study was to examine the effects of daily oral administration of doxy through oral gavage on Achilles tendon healing and biomechanical properties post-surgical repair. We hypothesize that Achilles tendon transection is associated with increased collagenase activity, and that oral administration of doxy through oral gavage could achieve appropriate systemic therapeutic levels in vivo resulting in improved Achilles tendon repair mediated through the inhibition of MMP activity. We also explored the effects of limited (1 week) versus extended (4 week) administration of doxy in order to ascertain a therapeutic time frame for the efficacy of doxy treatment for Achilles tears in a rat injury model.

METHODS

After IACUC approval, 100 male Sprague-Dawley rats (weight, 300–400 g) were obtained for this study. Each animal underwent surgical transection of the Achilles tendon alternating between right and left leg as site of injury. The rats were anesthetized using a 3% isoflurane with 1 L O_2 flow rate. Incision was made over the posterior aspect of the hind leg and sharp dissection of the calf exposed the Achilles tendon. The Achilles tendon was then completely transected in the midportion using a #15 scalpel blade and left unrepaired. The skin incision was repaired with 4-0 vicryl and animals were allowed to return to normal activity.

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Phase I—Measurement of Collagenase Activity

Levels of collagenase activity were measured in uninjured and in transected Achilles tendons. Forty rats had Achilles tendon transections as described above, and tendons were harvested from control (Day 0) and at 4, 8, 24, 48, 96 h, 2, 4, 6, and 8 weeks post-transection ($n = 4$ per time point). Total protein was extracted from harvested tendon tissue using a protein prep kit (Qproteome Mammalian Protein Prep Kit, Qiagen, Inc., Valencia, CA) and collagenase activity was determined by fluorescence measurement of collagen degradation using the Type I collagenase activity assay kit (Millipore ECM485), with type I collagenase (MMP-8) as a control enzyme.

Phase II—Collagenase Inhibition

Doxycycline hyclate (MP Biomechanics LLC, Solon, Ohio) was administered to examine its collagenase inhibition in vivo. Twelve rats were treated with 10 mg/kg of doxy daily through oral gavage, beginning 1 day prior to transection. These rats then had Achilles tendon transections as described above, and tendons were harvested at 96 h, 2 and 4 weeks post-injury. MMP activity in tendons was compared to the Phase I (no treatment) levels at corresponding time points. In order to ascertain the systemic dosage of doxy via oral gavage, serum was obtained immediately after euthanasia using cardiac puncture and analyzed via Kirby Bauer Agar Well Diffusion Assay. The diameter of the zone of inhibition was plotted against the antibiotic concentration against Staphylococcus aureus [ATCC 25923].

Phase III—Limited Versus Extended Duration of Doxy Administration

We examined the effect of doxy administered for a limited duration (1 week) prior to surgical repair versus extended duration (up to 4 weeks) post-tendon surgical repair. On Day 1, thirty-two rats were started on a daily administration of 10 mg/kg of doxy through oral gavage. A control group ($n = 16$) was given water by oral gavage. On Day 0, all rats sustained Achilles tendon transections as follows: the Achilles tendon midportion was marked with two 6-0 nylon suture ties and the tendon was completely transected using a #15 scalpel blade, leaving the unrepaired tendon ends marked. The skin incision was repaired and animals were allowed to return to normal activity. On Day 7, Achilles tendon was remobilized by removal of tissue adhesions, tissue callus was resected and tendon end ties were removed. The tendon ends were re-approximated and repaired with a modified Mason–Allen stitch using 4-0 vicryl suture. Animals in the limited duration group had doxy treatment suspended on Day 7 (day of surgical repair), whereas animals in the extended administration group continued to receive doxy daily after surgical repair up 4 weeks. Tendons harvested at 2 or 4 weeks post-injury were analyzed for histological evaluation ($n = 4$ per group), and tendons harvested at 3 weeks post-injury were analyzed for biomechanical properties ($n = 8$ per group).

Histological Analysis

Tendons were fixed in buffered formalin, embedded in paraffin, sectioned and stained with Mallory's trichrome or picrosirius red for collagen fiber analysis (AML Labs). Histological images of the Mallory trichrome samples were acquired at 100× and evaluated for collagen fiber orientation by a blinded observer trained in tendon histology and morphometry. Mallory trichrome slides were scored for collagen fiber orientation using a semi-quantitative grading system ($n = 4$).[25,26] The evaluation system was based on a grade of 0–3 according to the degree of collagen orientation, with lower grade corresponding to better histological result.[25,26] Picrosirius red slides were imaged under polarized light and acquired at 100×.

Biomechanical Testing

Biomechanical testing of the repaired tendons was performed on samples from three groups: (1) No doxy ($n = 8$), (2) Limited doxy ($n = 7$), (3) Extended doxy ($n = 8$). All dissections and uniaxial tensile testing were performed in a blinded fashion with respect to the treatment group, as previously described.[27] Briefly, a pre-load of 0.5 N was applied to the specimens, and the dimensions (length, width, depth) of the specimen were measured using digital calipers. The tendon was then subjected to tensile extension at a strain rate of 0.25%/s until failure and the resultant load was recorded. Sample stiffness was assessed from best fit of the linear portion of the load-displacement curve. The ultimate tensile strength, corresponding maximum load at failure, and elastic toughness were computed.

Sample cross sectional area was computed based on rectangular geometry and dimensions measured using digital calipers. Accuracy and resolution of the caliper-based measurements were performed using dedicated rat Achilles specimens ($n = 5$). Samples were mounted in the grips and dimension accuracy was determined from digital images (Logitech C920, 3MP). Resolution was estimated from maximum variability of 10 repeat measures on each sample. Caliper measurement technique has a 0.3 mm resolution and >95% repeatability. Length and width measurements have an accuracy of $93\% \pm 5\%$ and $90\% \pm 4\%$, respectively.

Statistical Analysis

Statistical analysis of MMP activity post-injury was analyzed with Kruskal–Wallis test and Dunn's multiple comparison test. Effects of doxy on MMP activity was analyzed with two-way ANOVA and Tukey HSD post hoc test. Histological grades were analyzed with two-way ANOVA and Bonferonni multiple comparison test. Biomechanical parameters were analyzed with one-way ANOVA and Tukey HSD post hoc test. Statistical analysis was performed with Statistica (Stat Soft, Inc) or GraphPad Prism, with $p < 0.05$ considered significant.

RESULTS

MMP-8 activity was measured in uninjured control tendons vs. injured tendons up to 8 weeks post-transection. At early time points post-injury (4, 8, 24, 48, and 96 h), MMP activity levels in the injured group were comparable to control group. An increasing trend in MMP activity levels was observed in transected tendons compared to uninjured tendons from 2 to 6 weeks post-injury, with peak activity levels observed at 4 weeks post-injury ($p < 0.0001$, Fig. 1). The peak in MMP activity reached at 4 weeks post-injury declined to approximately 2.5× the baseline activity levels measured in the uninjured control group by 8 weeks.

Daily administration of doxy via oral gavage significantly diminished MMP activity in the Achilles tendon

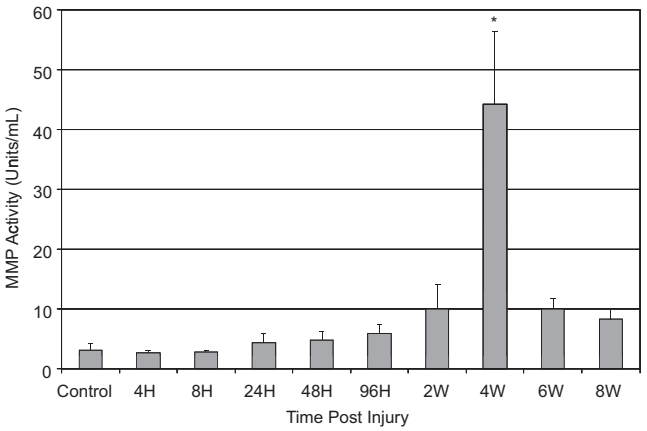

Figure 1. MMP activity in control or injured and repaired Achilles tendons up to 8 weeks post-injury/repair. $^{*}p < 0.05$ versus all other post-injury time points.

post-injury (Fig. 2). MMP activity in doxy treated group were 17% and 32% lower than untreated groups at 96 hours and 2 weeks post-injury, respectively (Fig. 2). After 4 weeks post-injury, MMP activity was found to be ~60% lower in the doxy treated group versus the untreated group ($p < 0.0005$, Fig. 2). Serum levels of doxy were found to be 6 μg/ml, which is within the clinically relevant dose of 1.8–9.4 μg/ml.[28]

Collagen fiber deposition was present in all groups examined histologically starting at 2-week time point (Fig. 3). There was a qualitative increase in collagen deposition (MTC) at 4 weeks relative to 2 weeks in all groups. Control tendons were characterized by a disorganized collagen orientation with some gaps between the proximal and distal portions in the region of transection. Collagen fiber orientation exhibited a trend for increased organization with increasing duration of doxy within each time point. Extended doxy at 4 weeks exhibited improved fibril alignment relative to limited or no doxy groups (Fig. 3). This morphological improvement was associated with decreasing histological grade trend at both 2- and 4-week time points (Fig. 4). Picrosirius red images under polarized light exhibited a mesh like organization in the no doxy groups at 2 weeks. Collagen fibrils showed improved organization and increased density in extended doxy groups at 2 and 4 weeks. Mixed orientation was seen in the limited doxy groups at both time points (Fig. 3). There was no evidence of cartilage formation or calcification in any of the samples examined histologically (Fig. 3).

The cross sectional area of the repaired tendons in animals treated with limited or extended duration of doxy was comparable to that in the no-doxy group. (No doxy: $0.14 \pm 0.05\,cm^2$; Limited: $0.13 \pm 0.03\,cm^2$; Extended: $0.14 \pm 0.06\,cm^2$; $p > 0.5$). Doxy treatment for extended duration resulted in significantly increased biomechanical properties of the Achilles tendon (Fig. 5). The maximum load at failure was significantly higher in Extended Doxy group vs. no doxy (21.8 ± 6.3 N vs. 32.0 ± 9.6 N, $p = 0.03$). This resulted in corresponding significant increases in the ultimate tensile strength ($p = 0.04$, Fig. 5). The elastic toughness was significantly greater in extended doxy group relative to no doxy (125 ± 48 mJ vs. 74 ± 40 mJ, $p = 0.03$; Fig. 5). Similarly, the tensile stiffness of tendons in the extended doxy group were significantly greater than the no doxy group (5.67 ± 1.6 N/mm vs. 7.76 ± 2.0 N/mm, $p < 0.05$). Similarly, we observed an increasing trend in elastic modulus of tendons in extended doxy group, relative to no doxy (6.59 ± 2.37 MPa vs. 8.95 ± 4.7 MPa). Administration of doxy for limited duration exhibited a trend of increased biomechanical properties compared to no doxy groups; however these increases were not statistically significant (Fig. 5).

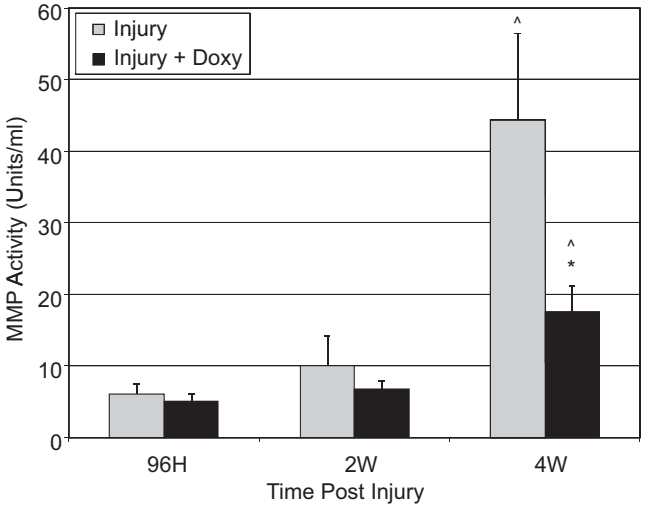

Figure 2. MMP activity in transected and repaired Achilles tendons up to 4 weeks post-injury with or without oral administration of doxy. $^{*}p < 0.05$ versus injury group within time point; $^{\wedge}p < 0.05$ versus 96H time point.

DISCUSSION

The goal of this study was to examine the effects of doxy administration by oral gavage on Achilles tendon healing and biomechanical properties post-surgical repair. Our results indicate that the use of doxy improves Achilles tendon repair quality and biomechanical properties, mediated in part by diminished Type I collagenase (MMP-8) levels in vivo. Our findings indicate that a temporal delay exists between Achilles tendon transection and associated increases in MMP-8 activity in situ. Significant increases in MMP activity post-injury were found to occur at 4 weeks post-injury, though levels well above baseline control activity were observed from 2 to 8 weeks post-injury. This temporal relationship provides some insight into identifying treatment window opportunities for effective Achilles tendon repair, and provides guidance for optimal administration of doxy as a MMP inhibitor.

Our findings demonstrate that daily oral administration of doxy can inhibit MMP-8 activity in the

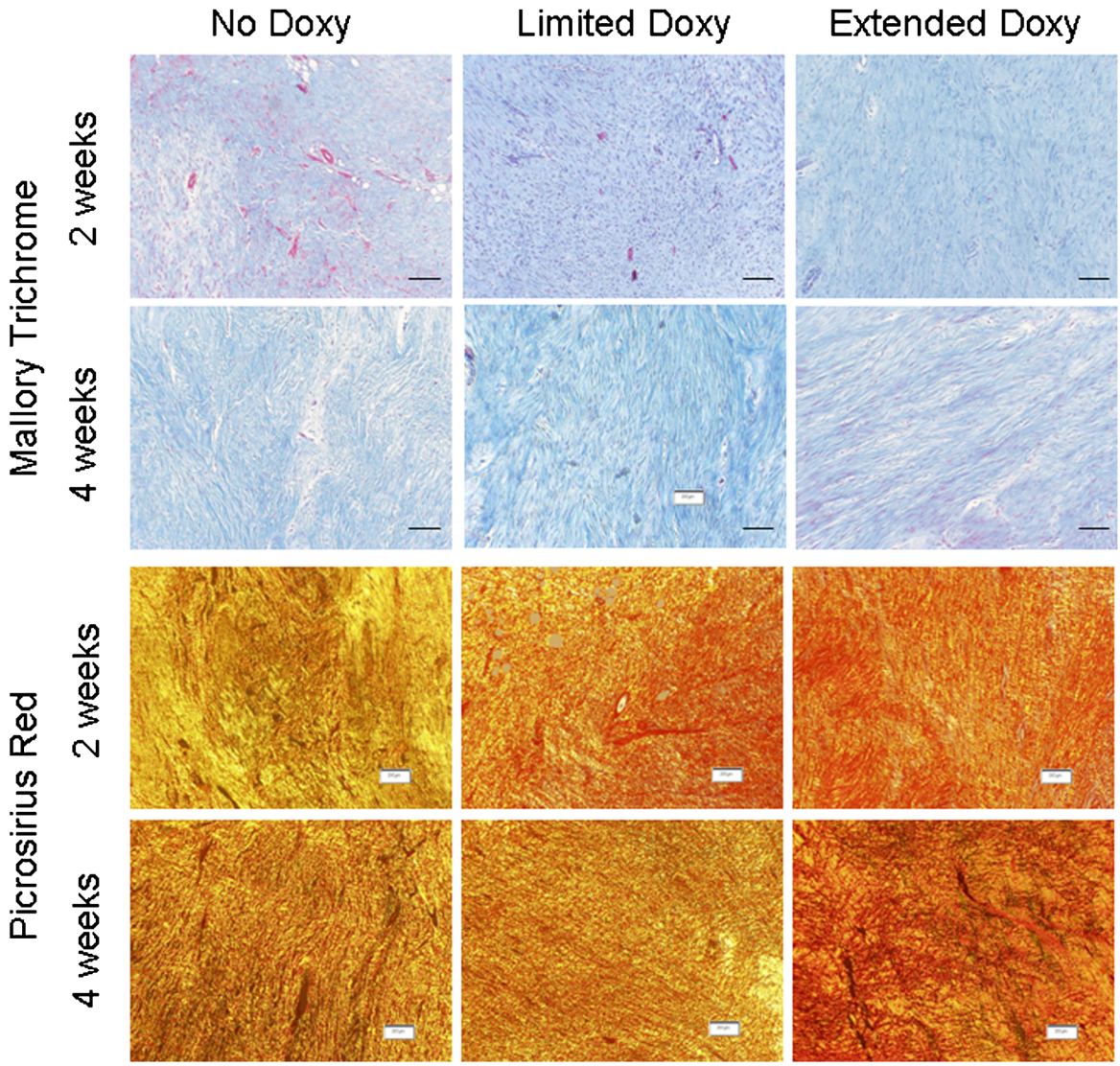

Figure 3. Histological evaluation of injured and repaired Achilles tendons at 2 or 4 weeks post-injury. Specimens were collected from animals receiving limited doxy treatment, extended doxy treatment or no treatment daily via oral gavage. Sections (top panel) were stained with Mallory trichrome or picrosirius red (bottom panel) (100×, scale bar = 200 μm).

Achilles tendon induced by tendon transection injury. Continuous oral administration of doxy for 2 weeks yielded moderate decrease in MMP activity level, though 4 weeks of continuous administration was needed to achieve significant inhibition in MMP activity in situ (Fig. 2). The limited efficacy of doxy after 2 weeks may be in part related to the delay observed in MMP activity post-injury. While doxy can effectively inhibit MMP activity, this inhibition is best observed when injury-induced MMP levels had peaked at 4 weeks (Fig. 1).

Oral administration of doxy for 4 weeks was associated with increased collagen deposition and improved fibril alignment (Fig. 3). Our findings suggest that doxy administration for a limited duration (1–2 weeks), ending prior to the time point associated with inhibition of MMP activity (3–6 weeks) was not associated with significant improvement in collagen alignment or tendon structure. Consequently, doxy administration resulted in ameliorated development of excessive fibrosis at time point coincident with significant inhibition of MMP activity.

Administration of doxy for extended duration had significant improvement on biomechanical properties of the Achilles tendon (Fig. 5). Interestingly, doxy administration didn't alter the cross sectional area of the repaired tendons in the no doxy versus limited or extended doxy groups. Since significant swelling is usually associated with tendon transection and repair, doxy did not differentially modulate swelling in the Achilles tendon. Consequently, significant increases in the ultimate tensile strength and elastic modulus in the extended treatment group may be due to improved tissue reorganization, while maintaining similar cross sectional areas to the no doxy group. Interestingly, the limited doxy group didn't exhibit improved histological

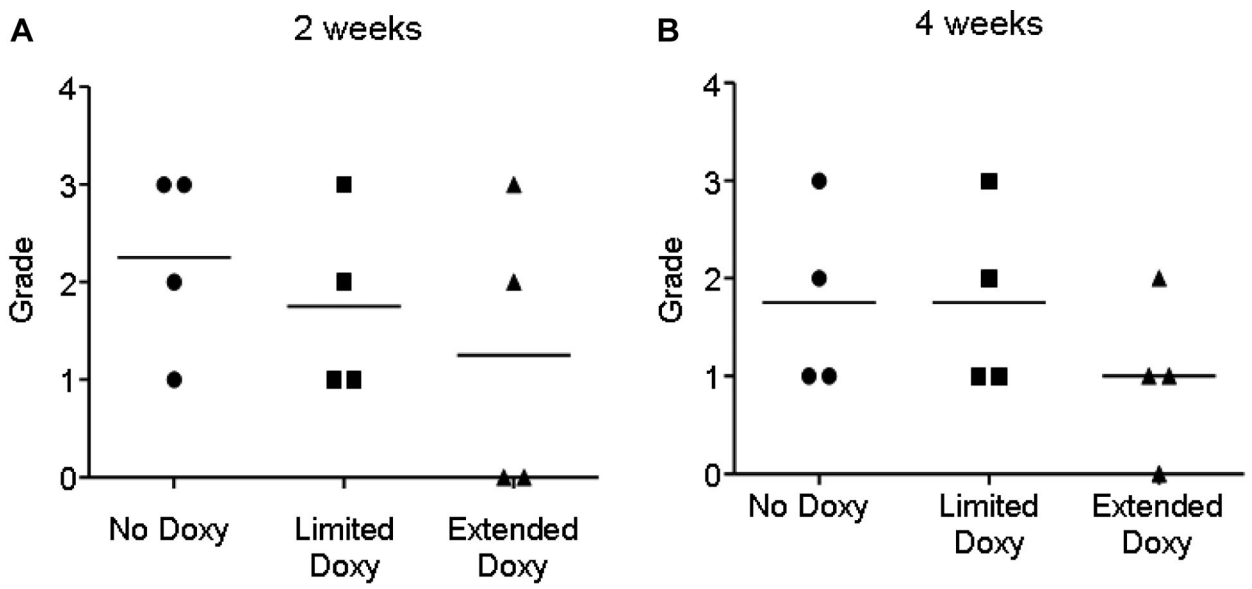

Figure 4. Scatter plot and average grade for collagen fiber orientation evaluated in histological sections stained with Mallory trichrome.

or biomechanical properties versus no doxy groups, further supporting the notion that inhibition of MMPs at peak activity levels is a key regulator of improved biomechanical properties.

Our findings indicate that clinically relevant serum levels of doxy can be obtained via daily oral gavage up to 4 weeks, confirming that oral administration of an MMP inhibitor can provide an efficacious route of

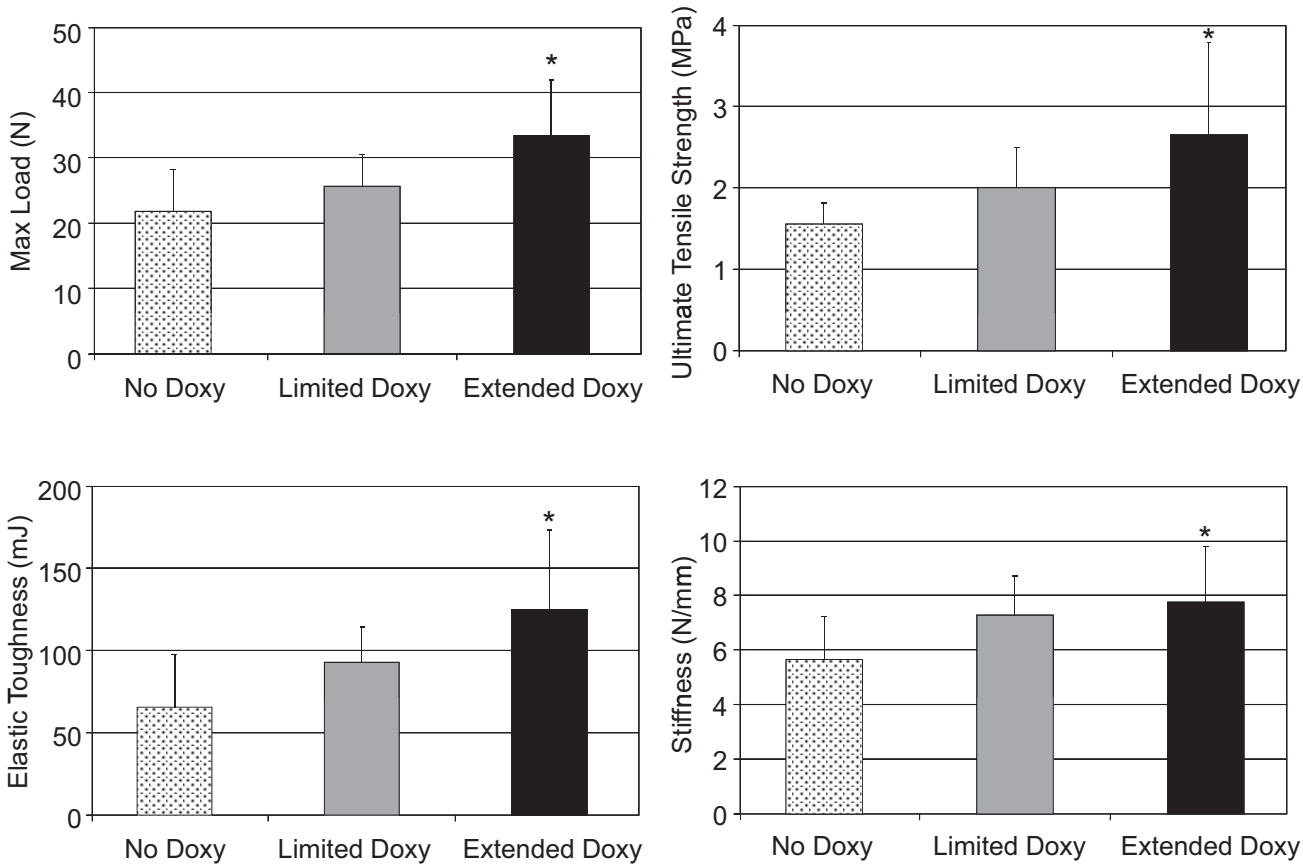

Figure 5. Biomechanical evaluation (max load, ultimate tensile strength, elastic toughness and stiffness) of Achilles tendons from limited, extended or no doxy groups. $^{*}p < 0.05$ versus no doxy.

administration in a small pre-clinical animal model. The 4-week duration of oral administration of doxy needed to observe significant inhibition of MMP activity in transected Achilles tendon may be in part dependent on low absorption of the medication and/or limited vascularization of the repaired Achilles tendon. Moreover, patients with ruptured Achilles tendons have been shown to exhibit elevated systemic levels of MMPs up to 2 years post-injury,[13] suggesting that extended administration may be needed to counteract long-term MMP levels due to Achilles injury.

In the current study, extended duration of drug delivery up to the time point of peak MMP-8 activity was necessary to achieve enhancement in tendon repair. This finding suggests that optimized long-term delivery is needed for improved Achilles repair, potentially due to limited vascularity in the tendon-to-tendon repair site in the Achilles. A previous study comparing short-term delivery of doxy systemically in drinking water versus locally with coated sutures demonstrated that both delivery systems improved suture pull out strength versus no doxy controls.[22] In a rotator cuff repair model, a local injection of the broad spectrum MMP inhibitor α2M was associated with increased collagen organization in the healing tendon-to-bone enthesis, though no structural improvement in biomechanical properties were observed.[29]

Our findings on limited duration administration of doxy demonstrate neither significant impairment nor improvement in the Achilles tendon. In a study by Pasternak et al.[23] systemic administration of doxy via drinking water up to 14 days post-injury significantly impaired the spontaneous healing of transected unrepaired Achilles tendons, potentially by delaying the removal of the callus associated with spontaneous healing. Whereas, Bedi et al.[24] demonstrated that oral administration of doxy through drinking water resulted in improved healing and biomechanics of surgically torn and repaired rotator cuff tendons at 14 days, but not at 28 days post-injury. While the effects of doxy in these two studies appear to be contradictory, this may be due to differences in repair responses of Achilles versus Rotator cuff injuries. Moreover, the examination of spontaneous repair versus surgical repair may contribute to these differences. It should be noted that the dose of doxy used in the current study was significantly smaller (10 mg/kg/day) relative to the aforementioned studies (100–130 mg/kg/day).[23,24] Overall, these findings highlight the difficulty in achieving optimal timing of delivery and efficacy of improved tendon repair with MMP inhibitors. Consequently, doxy delivery in our study for a duration covering the peak activity of MMP (4 weeks) but not prior to that (2 weeks) resulted in significant inhibition of MMP levels, amelioration of fibrosis development and improved biomechanical function.

To our knowledge, this study is the first to demonstrate that Achilles tendon transection results in elevated levels of MMP-8 in situ, and that inhibition of MMP-8 activity by doxy is associated with improved Achilles tendon repair and elastic biomechanical properties. Our findings on increased MMP activity using Achilles transection injury model are consistent with clinical studies of Achilles rupture in patients.[11,12] While our findings indicate that doxy inhibits injury associated MMP-8 activity, other proteases, such as MMP-9 or MMP-13 may also be modulated by doxy treatment.[24,30] The effects of doxy on other Type I collagenases (MMP-1 or MMP-13) or other proteases were not explored. It is also unknown if doxy modulates activity of endogenous TIMPs, known to be regulated in tendon injury.[8,11–13]

In the current study, complete inhibition of MMP-8 activity was not observed, although a significant reduction was achieved and found to be sufficient in augmenting Achilles tendon repair. We hypothesize that some degree of MMP-8 activity may be needed in the remodeling phase of tendon repair and that complete obliteration of MMP activity may interfere with improved repair. Though it is unclear if biological modulation of endogenous MMP activity down to basal levels is necessary to further augment healing after repair.

Some limitations of this study include the use and translation of our findings from a rat model to clinical human situation. In this study, administration of doxy began one day prior to tendon transection to ensure that serum levels of doxy would be clinically relevant at the time of surgery. While prophylactic doxy administration for tendon injury prevention is an unlikely mode of administration, patients may potentially receive the medication after sustaining a tear injury, but prior to surgical repair. The current study also surgically created a "clean cut" tendon transection using a scalpel, whereas clinical Achilles tendon ruptures are typically traumatic with shredding or "mop ends" morphology, and does not yield the same clean tear that was used in this study. It is unknown if a more severe clinical injury would benefit from doxy administration to the same level as in our induced injury model. In addition, the current study demonstrates observable benefits of doxy treatment on the biomechanics of Achilles tendon at a single early time point during the healing response. Future studies will investigate the effects of MMP inhibition at longer time points to ascertain if the observed benefits are sustained.

In summary, our findings indicate that treatment with oral doxy can provide a therapeutically relevant serum concentration and inhibits the increases in MMP-8 activity associated with Achilles tendon transection. Extended delivery of doxy resulted in improved tendon structure and biomechanical properties when compared to no doxy or limited duration treatment. These findings suggest that MMP inhibitors may be optimized for ameliorating development of fibrosis and improve the quality of repair after Achilles tendon ruptures.

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