

# Therapeutic Effects of Doxycycline on the Quality of Repaired and Unrepaired Achilles Tendons

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**Background:** Achilles tendon tears are devastating injuries, especially to athletes. Elevated matrix metalloproteinase (MMP) activity after a tendon injury has been associated with deterioration of the collagen network and can be inhibited with doxycycline (Doxy).

**Hypothesis:** Daily oral administration of Doxy will enhance the histological, molecular, and biomechanical quality of transected Achilles tendons. Additionally, suture repair will further enhance the quality of repaired tendons.

**Study Design:** Controlled laboratory study.

**Methods:** Randomized unilateral Achilles tendon transection was performed in 288 adult male Sprague-Dawley rats. The injured tendons were either unrepaired (groups 1 and 2) or surgically repaired (groups 3 and 4). Animals from groups 2 and 4 received Doxy daily through oral gavage, and animals from groups 1 and 3 served as controls (no Doxy). Tendons were harvested at 1.5, 3, 6, and 9 weeks after the injury ( $n = 18$  per group and time point). The quality of tendon repair was evaluated based on the histological grading score, collagen fiber orientation, gene expression, and biomechanical properties.

**Results:** In surgically repaired samples, Doxy enhanced the quality of tendon repair compared with no Doxy ( $P = .0014$ ). Doxy had a significant effect on collagen fiber dispersion, but not principal fiber angle. There was a significant effect of time on the gene expression of MMP-3, MMP-9 and TIMP1, and Doxy significantly decreased MMP-3 expression at 9 weeks. Doxy treatment with surgical repair increased the dynamic modulus at 6 weeks but not at 9 weeks after the injury ( $P < .001$ ). Doxy also increased the equilibrium modulus and decreased creep strain irrespective of the repair group. Doxy did not have a significant effect on the histology or biomechanics of unrepaired tendons.

**Conclusion:** The findings indicate that daily oral administration of Doxy accelerated matrix remodeling and the dynamic and equilibrium biomechanics of surgically repaired Achilles tendons, although such enhancements were most evident at the 3- to 6-week time points.

**Clinical Relevance:** The inhibition of MMPs at the optimal stage of the repair process may accelerate Achilles tendon repair and improve biomechanical properties, especially when paired with surgical management.

**Keywords:** Achilles tendon; doxycycline; MMP inhibition

Achilles tendon tears are devastating injuries, especially to athletes, with unpredictable outcomes with respect to return to play/function.<sup>25,30</sup> Several studies have suggested that non-operative treatment may result in outcomes similar to those of surgical repair and that early functional rehabilitation may be the key for both treatment options.<sup>18,35,47</sup> However, these studies also suggested that the rerupture rate after non-operative management exceeds that of operative care.<sup>18,35,47</sup>

Tendon repair differs based on the tendon interface, anatomic location, load type, and relationship to the joint affected. The study of tendon repair is further complicated by the unknown pathogenesis of most tendinopathies. Achilles tendon repair is initiated via 2 pathways and involves 3 phases.<sup>3,33,45</sup> The intrinsic pathway (intratendinous healing led by host tendon cells) results in strong, more elastic tendons. The extrinsic pathway, led by inflammatory and stem cells recruited to the injury site, results in a healing callus and scar tissue formation, contributing to reduced strength and elasticity and thus increasing the likelihood for reruptures. The first inflammatory phase of repair is characterized by increased vascular permeability and inflammatory cell influx (neutrophils, macrophages),

causing the release of chemotactic agents to recruit blood vessels, fibroblasts, and tenocytes. During the second proliferative phase, fibroblasts form granulation tissue by depositing the extracellular matrix (ECM), initially rich in collagen type III (COL3). In the last remodeling phase, a matrix metalloproteinase (MMP)-mediated process replaces COL3 with collagen type I (COL1), and cellularity decreases. Fiber organization increases in a direction parallel to the long axis of the tendon.

MMPs are zinc-dependent enzymes that degrade the ECM and play important roles in Achilles tendon injuries, disease, and repair.<sup>8,13,16,20</sup> After a tendon rupture, elevated MMP activity has been associated with deterioration in the quality of the collagen network.<sup>19,22,23,28,39,42,43</sup> The levels of MMPs and endogenous tissue inhibitors of metalloproteinases (TIMPs) fluctuate throughout the repair process to regulate tissue remodeling. Their dysregulation is implicated in the pathogenesis of tendinopathies.<sup>#</sup> The tenocyte production of MMP-1 and MMP-3 underscores the potential for the non-lymphocyte-mediated cytokine production of proteases inducing local matrix changes.<sup>46</sup>

In a prior study of Achilles tendon lacerations, we have shown that systemic, daily oral administration of doxycycline (Doxy) for 4 weeks can inhibit local MMP activity and result in improved collagen fibril organization and biomechanical properties in surgically repaired tendons.<sup>24</sup> The activity of Doxy, a potent tetracycline MMP inhibitor, is mediated by both direct and indirect mechanisms of action. Doxy has been used in tendon repair as well as in other clinical applications, including acne vulgaris, rosacea, pneumonia, endocarditis, Lyme disease, and anthrax.<sup>10,15,17,41</sup> MMP inhibitors and their activity may have different effects based on the timing of administration and type of repair model examined.<sup>2,37,38</sup> In our previous study, we demonstrated the benefit of extended administration compared with limited Doxy administration up to 2 weeks after an injury and repair. The duration of Doxy administration is an important factor in the resultant quality of tendon repair in part because MMP activity varies over time after an injury.<sup>24</sup>

The goal of this study was to examine the effects of daily oral Doxy administration on the repair quality of Achilles tendons after a midsubstance transection injury. We hypothesized that Doxy would enhance the histological, molecular, and biomechanical quality of transected Achilles tendons and that surgical treatment would further enhance

the quality of repaired tissue when compared with unrepaired tendons. The long-term effects (9 weeks) of daily systemic Doxy administration have not been examined previously in this model, and thus, this study investigated the temporal effects of Doxy treatment on the healing of surgically repaired and unrepaired Achilles tendons.

## METHODS

### Animal Study and Sample Harvest

This study was approved by the Institutional Animal Care and Use Committee at The Feinstein Institute for Medical Research. Adult, male 2- to 3-month-old Sprague-Dawley rats (weight, 350-400 g; N = 288) were used in this study. On day 0, each animal underwent surgical transection of the Achilles tendon, alternating between the right and left leg as the site of injury, with the contralateral Achilles tendon serving as an uninjured control. The rats were anesthetized with an intraperitoneal injection of ketamine (80 mg/kg) and xylazine (5 mg/kg) and maintained with 2% isoflurane (Baxter) with a 1 L/min O<sub>2</sub> flow rate. An incision was made over the posterior aspect of the hind leg, and blunt dissection of the calf exposed the Achilles tendon, which was then completely transected in the midportion using a No. 15 scalpel. The plantaris tendon was not preserved. The injured tendons were either left unrepaired (groups 1 and 2) or surgically repaired with a Mason-Allen stitch using 4-0 Vicryl sutures (Ethicon) (groups 3 and 4). The skin incision was closed with 5-0 Vicryl interrupted sutures, and animals were allowed to return to normal activity. Buprenorphine (0.05 mg/kg) was administered subcutaneously for analgesia in the postoperative period. Casts and dressings were not used, and the animals were allowed unrestricted activity after surgery.

One day after surgery (day 1), half of all surgically treated animals (n = 144) began a daily administration of 10 mg/kg of doxycycline hyclate (Doxy; MP Biomedicals) through oral gavage (groups 2 and 4; n = 72 each), and the remaining half (n = 144) served as controls (no Doxy; groups 1 and 3; n = 72 each). The Doxy dosage was established in our prior work with Achilles tendons.<sup>24</sup> At 1.5, 3, 6, and 9 weeks after the injury, the animals (n = 18 at each time point) were euthanized, and Achilles tendons were harvested for analysis.

For histological analysis (n = 4 per group), samples were harvested free of muscle and bone, fixed in 10% zinc formalin, and stored at room temperature until

#References 7, 12, 19, 22, 23, 28, 31, 39, 42.

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One or more of the authors has declared the following potential conflict of interest or source of funding: This study was funded in part by a Young Investigator Grant from the AOSSM (M.C.D., D.A.G., N.O.C.), the Department of Orthopaedic Surgery at the Long Island Jewish Medical Center, The Feinstein Institute for Medical Research, and the National Institute of Health (R01AR069668). The content is solely the responsibility of the authors and does not necessarily represent the official views of the National Institutes of Health.

TABLE 1  
Primer Sequences for Polymerase Chain Reaction<sup>a</sup>

Gene	Forward Primer Sequence	Reverse Primer Sequence
<i>COL1</i>	5'-GCTGAATCCTTCCGTGTT-3'	5'-AGGGAGGGGACTTATCTG-3'
<i>COL3</i>	5'-AGGCTTTGATGGACGCAATG-3'	5'-GCGGCTCCAGGAAGACC-3'
<i>MMP-1</i>	5'-AGTCATACAGTTTCCCCGT-3'	5'-GCCTCAGCTTTTCAGCCATC-3'
<i>MMP-3</i>	5'-ATGAACGATGGACAGATG-3'	5'-TGTGGAGGACTTGTAGACT-3'
<i>MMP-9</i>	5'-GCTATGGTTACACTCGGGCA-3'	5'-TGGCCTTTAGTGTCTCGCTG-3'
<i>MMP-13</i>	5'-TGTGACCCAGCCCTATCC-3'	5'-ACCCCTCCATAATGTCATACCC-3'
<i>TIMP1</i>	5'-CATGGAGAGCCTCTGTGGAT-3'	5'-TGTGCAAATTTCCGTTCCCTT-3'
<i>TIMP2</i>	5'-CGTTTGCAATGCAGACGTA-3'	5'-GATGGGGTTGCCATAGATGT-3'
<i>SCX</i>	5'-CGAAGTTAGAAGGAGGAGGGT-3'	5'-CGCTCAGATCAGGTCCAAAG-3'
<i>TNMD</i>	5'-GGACTTTGAGGAGGATGG-3'	5'-CGCTTGCTTGTCTGGTGC-3'
<i>TNC</i>	5'-GCTACTCCAGACGGTTTC-3'	5'-TTCCACGGCTTATTCAT-3'
<i>GAPDH</i>	5'-TGGAGTCTACTGGCGTCTT-3'	5'-TGTCATATTTCTCGTGGTTCA-3'

<sup>a</sup>Genes are defined/expanded in the text.

further processing. For biomechanical analysis ( $n = 8$  per group), samples were harvested with a bone and muscle margin, wrapped in saline-soaked gauze in individual specimen cups, and frozen at  $-20^{\circ}\text{C}$ . For gene expression analysis ( $n = 6$  per group), samples were harvested free of muscle and bone, and the midline portion of the tendon was excised for analysis, submerged in RNAlater (Ambion, Thermo Fisher Scientific), and stored at  $-20^{\circ}\text{C}$ .

## Histological Analysis

Tendons were fixed in buffered formalin; embedded in paraffin; cut into 10- $\mu\text{m}$  sections; and stained with hematoxylin and eosin (H&E), Mallory trichrome, or Picrosirius red staining kits (American MasterTech). Histological images of the H&E and Mallory trichrome slides were acquired with a BH-2 microscope and DP72 camera (Olympus) and evaluated for the quality of tendon repair by a blinded observer trained in tendon histology and morphometry. Tendon histology was graded using a modified tendon grading scale that expanded on previously established scales, including additional variables involved in tendon healing.<sup>4,6,9,14,21,32,36,40</sup> Samples were graded in the following categories: cellularity (inflammatory cells), collagen fiber organization, vascularity/angiogenesis, cell shape (fibroblastic changes), presence of ectopic cartilage, and presence of granulation tissue. Each variable was evaluated on a scale of 0 to 3, with grade 0 being normal and having no abnormalities, grade 1 having less than 25% abnormalities, grade 2 having less than 50% abnormalities, and grade 3 being the most disorganized poorly healed tissue and having more than 50% abnormalities.

Images of Picrosirius red-stained tendon sections were taken using polarized light microscopy (BH-2 microscope and DP72 camera), with the tendon fibers aligned vertically, and used to quantitatively evaluate collagen fiber organization using fast Fourier transform (FFT).<sup>1</sup> Briefly, the original image was transformed from real space to frequency space using FFT, from which a pixel intensity plot against the angle of acquisition was generated. The

magnitude of fiber alignment in the original image was indicated by the height and overall shape of the intensity frequency plot. The principal axis of orientation was determined from the position of the peak in the intensity frequency plot. The lower and upper limits of fiber angles were defined as the angles at which the intensity dropped to 50% of the peak intensity. The range of fiber dispersion was computed as the range between the lower and upper limits of fiber angles.

## Gene Expression Analysis

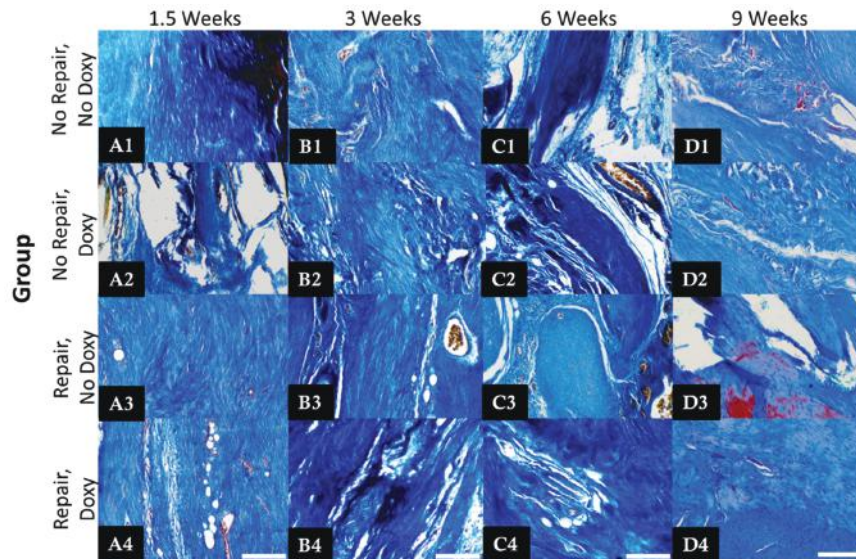
Rat Achilles tendons were homogenized using the gentle MACS Dissociator and M Tubes (Miltenyi Biotec). RNA extraction and purification were completed with the RNeasy Mini Kit (Qiagen) and RNase-Free DNase Set (Qiagen). cDNA was synthesized with the iScript cDNA Synthesis Kit (Bio-Rad) and T100 Thermal Cycler (Bio-Rad). Gene expression analysis was performed using the LightCycler 480 (Roche) with iQ SYBR Green Supermix (Bio-Rad) following standard protocols.

Analysis of gene expression was performed in study samples and normal uninjured tendons for the following (Table 1): *COL1* and *COL3* (components of the tendon ECM),<sup>26,29,48</sup> *MMPs* (1, 3, 9, 13), and *TIMPs* (1, 2). The expression of scleraxis (*SCX*; marker of neotendon formation),<sup>34</sup> tenomodulin (*TNMD*; marker of tendon differentiation and maturity), and tenascin C (*TNC*; regulator of collagen fibrillogenesis)<sup>5,29</sup> was also measured. The expression of glyceraldehyde 3-phosphate dehydrogenase (*GAPDH*) was used as a housekeeping gene. The fold change of gene expression in the study samples was normalized and the expression levels measured in uninjured normal tendons using the Pfaffl method ( $\Delta\Delta C_t$  method).<sup>27</sup>

## Biomechanical Analysis

Frozen Achilles tendons were thawed in phosphate-buffered saline at room temperature. Each specimen was mounted vertically between 2 pneumatic clamps in





**Figure 1.** Representative images of Achilles tendon sections stained with Mallory trichrome showing matrix organization. Tendons were collected from 4 experimental groups: group 1 (no repair, no Doxy), group 2 (no repair, with Doxy), group 3 (repair, no Doxy), and group 4 (repair, with Doxy) at (A1-A4) 1.5 weeks, (B1-B4) 3 weeks, (C1-C4) 6 weeks, and (D1-D4) 9 weeks after a laceration injury. Scale bar = 0.2 mm.

a materials testing machine (Model #5566; Instron), equipped with a 10-N load cell, and submerged in a phosphate-buffered saline bath for testing. The sample was preloaded to 0.1 N, and the length, width, and depth were measured using digital calipers.<sup>24</sup> Two consecutive stress-strain relaxation ramps were applied, to an elongation of 5% and 10% of the initial length, at a rate of 0.1% per second. A relaxation period of 900 seconds was allowed after each tensile ramp. At the end of the equilibrium tensile test, the same sample was returned to 0% strain for 900 seconds to recover to its original configuration. The relaxation and resting time was selected based on preliminary tests showing that this time period was enough for samples to reach 95% of their equilibrium values. The sample was then subjected to cyclic loading between 1 and 5 N under a load control for 35 cycles at 0.1 Hz. Data from the equilibrium tensile tests were analyzed for the equilibrium tensile modulus ( $E_{ten}$ ), computed from the best fit of the stress-strain curve (see Appendix Figure A1, available in the online version of this article). Data from the last 10 cycles of cyclic tests were analyzed for viscoelastic properties, including the dynamic modulus and dynamic stiffness. Other viscoelastic mechanical properties (ie, hysteresis, creep strain) were also calculated (see Appendix Figure A2, available online). Viscoelastic properties are better indicators of functional mechanical recovery in tendons as this type of tissue exhibits viscoelasticity under dynamic loading. All computations were performed using MATLAB subroutines (MathWorks).

### Statistical Analysis

Data are presented as the mean  $\pm$  SEM. Based on the study design, the effects of 3 factors were considered—surgical repair (yes, no), Doxy (yes, no), and time (1.5, 3, 6, 9 weeks)—as well as interactions between factors. For histological

grading scores, the normal residuals were verified, and therefore, a comparison of effects was performed using 3-way analysis of variance with Tukey-Kramer adjustment (SAS; SAS Institute). Measures of collagen fiber alignment, gene expression, and biomechanical properties were also compared using 3-way analysis of variance. Additional comparisons were made for the effects of time and Doxy within unrepaired (group 1 vs group 2) or repaired (group 3 vs group 4) groups. Pairwise comparisons between groups were performed using Tukey honest significant difference post hoc tests (Statistica; StatSoft). In all analyses, the significance level was set at  $P < .05$ , and trends are noted for  $P < .1$ .

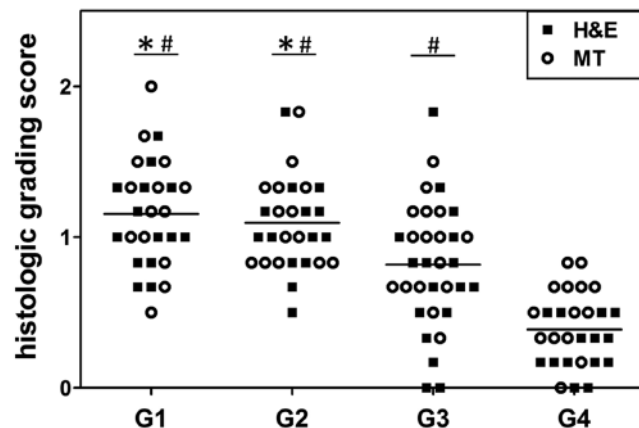
## RESULTS

### Histological Analysis

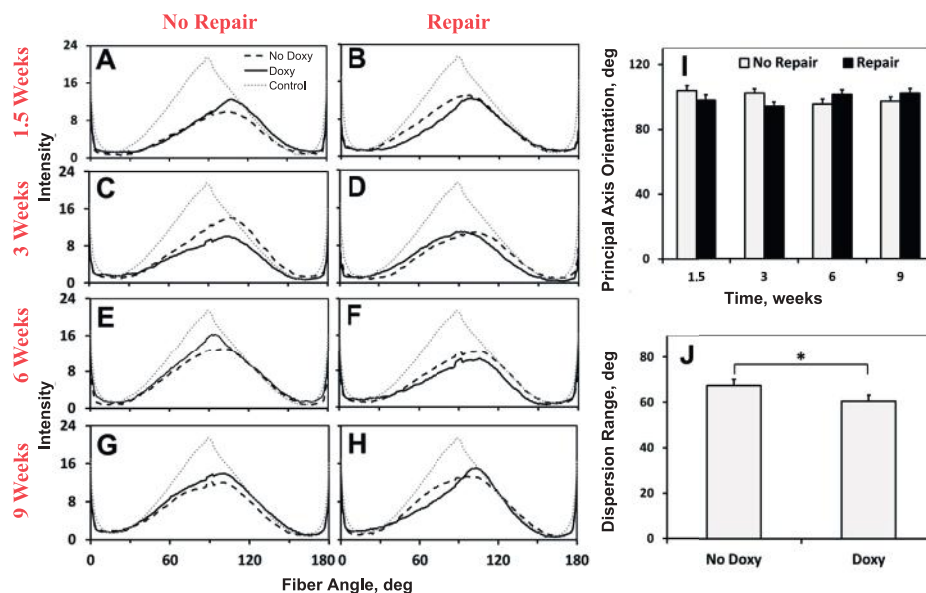
**Tendon Histological Grading Score.** Representative images of Achilles tendon sections stained with Mallory trichrome are presented in Figure 1. Tissue organization improved over time after transection, with tendons evaluated at 9 weeks exhibiting the most organized tissue structure at the repair site (Figure 1). At earlier time points, tissue had poor organization of the collagen network (Figure 1, A1-A4), but there was re-establishment of this network by 9 weeks (Figure 1, D1-D4). Tendons from unrepaired groups (groups 1 and 2) exhibited a more disorganized tissue structure at all time points, with increased vascularity and granulation tissue, when compared with groups that underwent suture repair (groups 3 and 4). Doxy improved tissue organization (groups 2 and 4) relative to the controls (no Doxy; groups 1 and 3). When comparing all groups across time, observations of superior fiber organization and the lack of vascularity or ectopic cartilage formation were made in group 4 tendons.

All 3 experimental factors (repair, Doxy, and time) had a significant effect on the histology score (repair:  $P < .0001$ ; Doxy:  $P < .005$ ; time:  $P < .0005$ ). Moreover, the interaction between repair and Doxy was found to be significant ( $P < .02$ ), although the interaction with time was not statistically significant. Therefore, pairwise comparisons of histological grading scores were performed for the 4 repair/Doxy combinations from all time points and are presented in Figure 2. Tendons in group 1 (no repair, no Doxy) exhibited the highest mean histological grading scores, indicative of the most disorganized matrix and poor healing, regardless of time after the injury. Suture repair of tendons significantly improved the histological grading score when compared with unrepaired tendons, both in the absence (group 1 vs group 3;  $P < .005$ ) and presence (group 2 vs group 4;  $P < .0001$ ) of Doxy administration. The mean score of unrepaired tendons was similar in Doxy-treated groups compared with their respective no-Doxy groups (group 1 vs group 2;  $P = .99$ ). In surgically repaired samples, the daily administration of Doxy enhanced the quality of tendon repair, as indicated by lower grading scores in group 4 samples compared with group 3 samples at all time points (group 3 vs group 4;  $P = .0014$ ). Group 4 (surgical repair with Doxy) had significantly lower histological grading scores irrespective of time when compared with all the other groups ( $P < .005$ ).

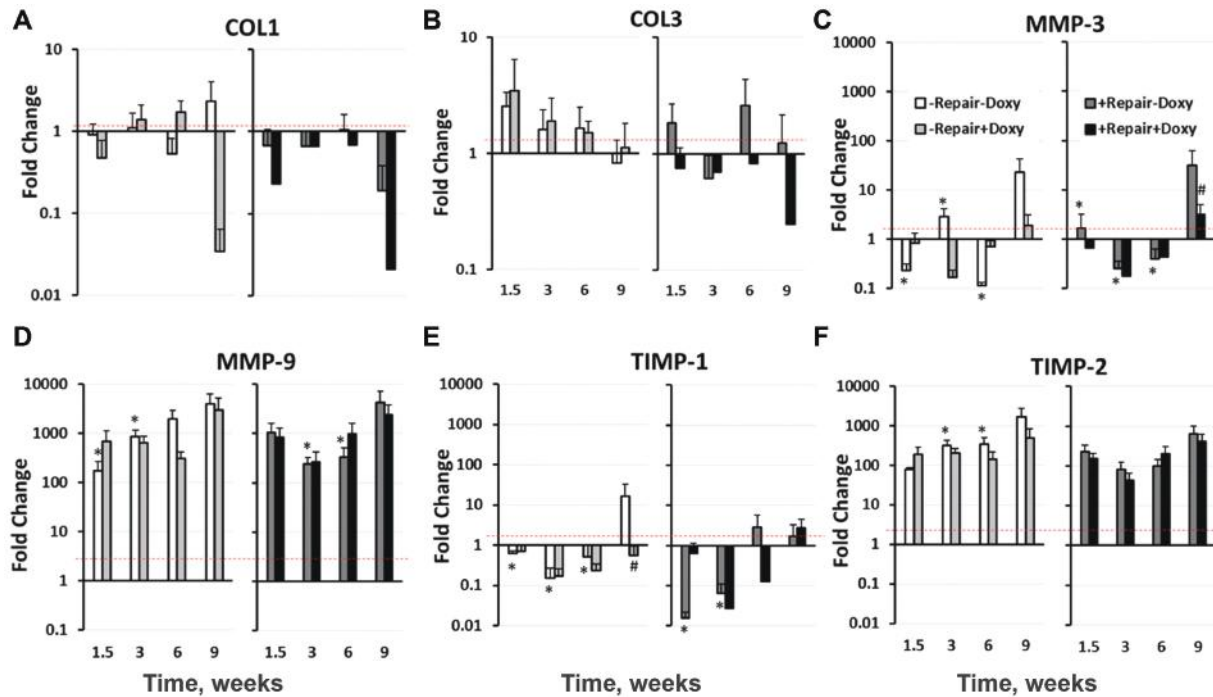
**Collagen Fiber Orientation.** The collagen fiber alignment of the samples in all groups was analyzed in sections stained with Picrosirius red (Figure 3). The frequency plot



**Figure 2.** Histological grading scores from hematoxylin and eosin (H&E; filled square) and Mallory trichrome (MT; open circle) staining for individual tendon samples from groups G1 through G4 collected at 1.5 weeks, 3 weeks, 6 weeks, and 9 weeks after injury. Scores range from 0 to 3, with 0 being normal and 3 being the most disorganized and poorly healed tissue. Bars represent the mean of the scores for individual groups (\* denotes  $P < .005$  compared to G3, and # denotes  $P < .005$  compared to G4).



**Figure 3.** Distribution of collagen fiber orientation in Achilles tendons at (A, B) 1.5 weeks, (C, D) 3 weeks, (E, F) 6 weeks, and (G, H) 9 weeks after a laceration injury in animals that were treated with Doxy (solid black line) or without Doxy (dashed black line) when compared with controls (dotted gray line). Tendon samples were compared between Doxy and no Doxy of (A, C, E, G) unrepaired groups and (B, D, F, H) repaired groups. (I) The mean peak angle of primary fibers and (J) range of fiber dispersion were also computed for each group. Doxy resulted in a smaller dispersion range across all time points and groups ( $P < .05$ ).



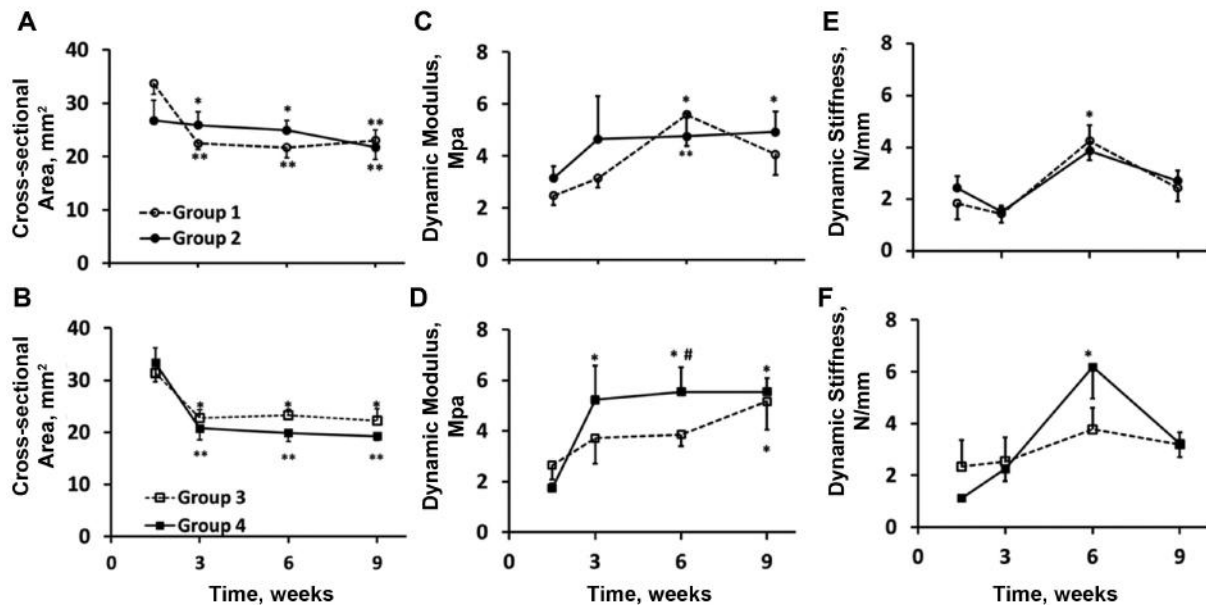
**Figure 4.** Relative fold change of gene expression measured by quantitative reverse transcription polymerase chain reaction of (A) collagen type I (COL1), (B) collagen type III (COL3), (C) matrix metalloproteinase (MMP)–3, (D) MMP-9, (E) tissue inhibitor of metalloproteinases 1 (TIMP1), and (F) TIMP2. Dashed lines indicate the expression of normal uninjured tendon samples. \* $P < .05$  denotes significance with time when compared with unrepaired or repaired groups (group 1 or group 3) at 9 weeks. # $P < .05$  denotes a significant effect of Doxy when compared with the no-Doxy group at the same time point.

of collagen fiber angles in control tendons exhibited a tall and narrow shape distribution, with a mean principal axis direction of  $87.9^\circ \pm 0.6^\circ$  (where x-axis =  $0^\circ$  or  $180^\circ$  and y-axis =  $90^\circ$ ). When compared with control samples, the principal direction of collagen fibers in injured samples was greater, and a broader range of fiber dispersion was observed (Figure 3, A–J). The principal fiber orientation of the samples in all groups was within  $5^\circ$  to  $15^\circ$  of the normal tendon fiber orientation. There was a significant interaction observed between repair and time for the principal direction ( $P = .029$ ) (Figure 3I). The mean principal fiber angle in unrepaired tendons decreased from  $104.0^\circ \pm 3.0^\circ$  at 1.5 weeks to  $97.3^\circ \pm 2.8^\circ$  at 9 weeks. In repaired tendons, we observed a different trend in which the mean principal fiber angle increased with time from  $98.0^\circ \pm 3.2^\circ$  to  $102.4^\circ \pm 2.8^\circ$ . Group 4 samples exhibited a principal fiber direction that was nearest to the normal tendon level ( $90^\circ$ ) at the 3-week time point. Although there was no significant effect of Doxy on the principal fiber angle, Doxy treatment had a significant effect on fiber dispersion, irrespective of repair or time. Doxy-treated samples had significantly lower fiber dispersion ( $61.1^\circ \pm 2.4^\circ$ ) compared with the no-Doxy groups ( $67.8^\circ \pm 2.3^\circ$ ) ( $P = .045$ ) (Figure 3J). At 6 and 9 weeks, group 4 samples exhibited the lowest fiber dispersion, and levels were comparable with those of a normal tendon ( $55.4^\circ \pm 2.9^\circ$ ).

### Gene Expression Analysis

The gene expression of tendons from the 4 experimental groups is reported as the fold change relative to expression levels measured in normal (uninjured) tendons (Figure 4). COL1 expression level had a decreasing trend with repair, irrespective of Doxy or time, relative to a normal tendon ( $P = .053$ ) (Figure 4A). COL3 also had a trend for decreasing the expression level with repair ( $P = .1$ ) (Figure 4B). There was a significant effect of time on the expression levels of MMP-3 ( $P = .041$ ), MMP-9 ( $P = .0009$ ), and TIMP1 ( $P = .004$ ). MMP-9 levels were lowest at 3 weeks and highest at 9 weeks, with intermediate levels observed at 1.5 and 6 weeks. MMP-9 levels at 9 weeks were significantly greater than at all other time points, irrespective of repair or Doxy ( $P < .01$ ). MMP-3 and TIMP1 levels increased with time, with the greatest levels observed at 9 weeks compared with the other time points (MMP-3:  $P < .01$ ; TIMP1:  $P < .016$ ). When examining the effect of Doxy within the repaired groups, it was found that MMP-3 expression decreased with Doxy at 9 weeks ( $P < .05$ ) (Figure 4C). A similar effect of Doxy on MMP-3 was observed within the unrepaired groups ( $P = .05$ ) (Figure 4C). There was also a significant decrease in TIMP1 expression with Doxy at 9 weeks in the unrepaired groups ( $P < .05$ ) (Figure 4E). There was no significant difference detected in the expression of TIMP2, SCX,





**Figure 5.** (A, B) Cross-sectional area, (C, D) dynamic modulus, and (E, F) dynamic stiffness of Achilles tendons at 1.5, 3, 6, and 9 weeks after a laceration injury. After the injury, the tendon was treated with Doxy or without Doxy and (A, C, E) without or (B, D, F) with suture repair. \*\* $P < .05$  and \* $P < .0001$  denote the significance level when compared within the same group at 1.5 weeks. # $P < .05$  denotes significance when compared with the respective no-Doxy group at the same time point.

*TNC*, and *TNMD* with respect to repair, time, or Doxy treatment (Figure 4F and Appendix Figure A3, available online). The expression levels of *MMP-1* and *MMP-13* were undetectable in many samples and thus could not be further evaluated.

### Structural and Biomechanical Analysis

The cross-sectional area (CSA) of repaired tendons decreased significantly with increasing time after the injury ( $P < .0001$ ) (Figure 5, A and B). In the unrepared groups, Doxy significantly reduced the CSA of tendons shortly after the injury ( $P < .05$  at 1.5 weeks after injury) and continued to affect the CSA at longer time points (Figure 5A). Suture repair had a similar effect on the CSA shortly after the injury (at 1.5 weeks) as well as at later time points ( $P < .05$ ) (Figure 5B). Although the administration of Doxy appears to have accelerated remodeling of the matrix, as indicated by a decreasing CSA, no significant effect of Doxy treatment was observed on the CSA in either unrepared or surgically repaired samples (Figure 5, A and B).

There was a significant effect of time ( $P < .0001$ ) and trend for the effect of Doxy ( $P = .07$ ) on the dynamic modulus. The dynamic modulus significantly increased over time in both unrepared and repaired groups (Figure 5, C and D). There was no significant effect of Doxy treatment in the unrepared groups (Figure 5C). Doxy treatment resulted in a significant increase of the dynamic modulus at 6 weeks after a laceration injury in surgically repaired samples ( $P < .001$ ) (Figure 5D). A similar trend for an increased dynamic modulus in group 4 was observed at 3

weeks. However, at 9 weeks after the injury, both groups had a similar dynamic modulus. Dynamic stiffness also had a significant effect of time, with the highest stiffness levels measured at 6 weeks compared with all other time points, independent of Doxy or repair ( $P < .0001$ ) (Figure 5, E and F).

The resulting creep strain measured during cyclic loading had a significant effect of Doxy treatment ( $P = .048$ ) and time ( $P < .0001$ ), irrespective of the repair group (see Appendix Figure A2, available online). A significant interaction between Doxy and time was also observed on creep strain ( $P = .04$ ). Creep strain was significantly lower in the Doxy-treated groups compared with the no-Doxy groups, with the most notable effects occurring at the 3-week time point ( $P = .027$ ). No significant effects of the evaluated variables were observed on hysteresis.

The equilibrium modulus had a significant effect of time ( $P < .0001$ ) and trend effect due to Doxy treatment ( $P = .065$ ), irrespective of the repair group (see Appendix Figure A1, available online). The equilibrium modulus was significantly higher at 6 weeks compared with the 1.5- and 3-week time points ( $P < .02$ ). Doxy treatment resulted in a greater equilibrium modulus compared with the no-Doxy groups ( $0.68 \pm 0.082$  MPa vs  $0.46 \pm 0.077$  MPa, respectively;  $P = .040$ ).

### DISCUSSION

The aim of this study was to examine the effects of the daily administration of Doxy through oral gavage on the quality of Achilles tendon repair without immobilization

or the use of a cast to determine the combined effects of Doxy and surgical repair. The quality of the tendon was examined at various stages of the repair process (as early as 1.5 weeks and up to 9 weeks after injury). The results presented here indicate that the daily administration of Doxy accelerated recovery in biomechanical properties (ie, greater equilibrium modulus, higher dynamic modulus and lower creep strain) as well as structural properties (ie, lower histological grading score and more physiological collagen fiber dispersion). Moreover, Doxy significantly reduced the expression of MMP-3 at the longest time points. Our findings suggest that the combination treatment of surgical repair and Doxy enhances tendon repair in a rat model of Achilles tendon lacerations. Thus, Doxy has the potential for use as an adjunct for the treatment of Achilles tendon tears to enhance the biomechanical function of suture-repaired Achilles tendons.

The results from the current study confirm and extend those from our previous work on the effects of systemic Doxy administration in Achilles tendon repair.<sup>24</sup> The time points in the current study were chosen based on our earlier work, which showed that healing is typically achieved in this model at approximately 6 weeks and that the greatest differences due to repair are typically observed in the 3- to 6-week time frame.<sup>24</sup> A 9-week time point was added to further ascertain the effect of long-term Doxy administration. We observed that Doxy accelerated the repair of Achilles tendons, as indicated by better histological grading scores and a higher dynamic modulus at 6 weeks after the injury, which represents a longer time point than the previously studied 4-week time point.<sup>24</sup> Interestingly, extending Doxy administration out to 9 weeks had mild effects on tendon quality in the measured outcomes. These findings suggest the administration of Doxy for a duration of 4 to 6 weeks to be most beneficial for tendon quality after surgical repair in this injury and repair model. Interestingly, observations of improved fiber organization, diminished MMP expression, and biomechanical enhancement in the Doxy groups began at the 3-week time point; however, the sustained effects were not appreciated until the 6-week time point.

While surgical repair of tendons resulted in better histology scores and collagen fiber organization when compared with unrepaired tendons (albeit both groups were without immobilization/casts), further enhancements of Doxy treatment on unrepaired tendons were not realized. Doxy administration in unrepaired tendons did not have a marked effect on biomechanical properties either, although the CSA was initially diminished at the early time point. The differential effect of Doxy on repaired and unrepaired tendons suggests that surgical repair is necessary for the MMP-dependent activity of Doxy to show efficacy. Indeed, surgical repair can enhance or accelerate MMP activity during tissue remodeling. We postulate that repair may regulate the MMP-dependent efficacy of Doxy by promoting synergy between Doxy-mediated and TIMP1-mediated inhibition of MMPs. Our results indeed suggest that surgical repair and Doxy lowered the expression of MMP-3, consistent with this hypothesis.

While the positive effects of Doxy on tendon repair found in this study are consistent with those in our prior study, they are however different from other studies showing that the systemic administration of Doxy had little effect on or even impaired tendon repair in rats,<sup>11,37</sup> although local delivery improved the strength of the Achilles tendon at the suture site.<sup>38</sup> The varying results might be caused by different levels of Doxy being administered in the various studies. We used 10 mg/kg of oral Doxy, whereas other studies have used 100 to 130 mg/kg.<sup>11,37</sup> Another factor that may have contributed to the differences was the method of obtaining mechanical properties of the Achilles tendon. The present study utilized a loading protocol that captured the viscoelastic and elastic behavior of tendons, whereas in previous studies, testing to failure was employed.<sup>11,37</sup> Tendons exhibit viscoelastic behavior, which is the result of a complex interaction between matrix constituents and fluids, interactions between various ECM components, as well as a viscoelastic solid matrix. Hence, viscoelasticity may be a more sensitive indicator of functional changes in biomechanical properties of Achilles tendons.

The variation in rehabilitation strategies after an injury and after repair may exert a sustained effect on tendon healing. In this study, animals were allowed to return to normal activity immediately after surgery. The effects of physiological loading on healing tendons are complex and may contribute to the observed findings of this study. However, optimal rehabilitation strategies after repair for tendons depend on the particular tendon environment and functional requirements. In this study, immobilization or casts were not used; thus, the animals were allowed to bear weight throughout the study. Clinically, patients who are treated conservatively for Achilles tendon tears are typically assigned a splint in plantar flexion for 2 weeks and then advised to wear a boot with partial weightbearing and early physical therapy for range of motion. This has been shown to have better functional outcomes than placing patients in a cast and results in similar clinical outcomes to surgical repair.<sup>47</sup> In a rat model, it was not practically possible to re-create the exact clinical scenario in this study. Moreover, the comparison of groups from unrepaired samples without casts/immobilization provided the most appropriate and direct comparator to determine the effect of surgical repair.

For this study, a routine histological grading scale was modified to include more categories of factors involved with tendon healing.<sup>4,6,9,14,21,32,36,40</sup> Normal tissue is more organized than healed injured tissue, which has implications in reduced strength and elasticity of the tissue because of scar formation. Tendons transfer loads from soft muscle to hard bone and are at a high risk of reinjuries and reduced functionality due to scarring.<sup>5</sup> We observed an improvement in the mean histology scores across time in all experimental groups, with group 4, which received the combination treatment (suture repair with Doxy), exhibiting the best mean scores closest to a normal tendon. When we focused on the collagen fiber organization score only, we noted that group 4 exhibited improvement in fiber organization earlier than the other groups and exhibited the best (closest to normal) collagen fiber dispersion across all time points and groups. This would suggest that the combination



treatment in group 4 (suture repair with Doxy) stimulates earlier remodeling of healing tendons. Additionally, our collagen fiber organization scoring was consistent with the FFT analysis of Picrosirius red-stained tissue, further validating the histological scoring system.

Changes in the expression levels of genes that are involved in tendon repair and remodeling were consistent with those reported in previous studies on Achilles tendon ruptures and repair.<sup>22,23,44</sup> Tendon repair is a complex process orchestrated by macrophages and fibroblasts and involves various cytokines and growth factors.<sup>44</sup> The increase in the gene expression of MMPs and TIMPs with longer times after the injury was also consistent with previous studies.<sup>22,23,44</sup> MMP-3 expression was elevated at the early stages of repair (up to 3 weeks after injury) and again at a later stage (at 9 weeks after injury), and Doxy markedly reduced it both in unrepaired and surgically repaired tendons (Figure 4E).

While the use of an animal model captures important aspects of tendon injuries and repair, it is important to keep in mind that the use of rats in this study does not capture the complete cause of the Achilles tendon injury seen in human patients. In the current study, the injury was created as a “clean-cut” laceration using a scalpel, whereas clinical Achilles tendon ruptures typically occur as acute trauma of chronically degenerated tendons, resulting in shredding or a “mop-end” shape. The morphological characteristics as well as the quality of tissue at the injury site could greatly affect the repair process. Another limitation of the current study was that biomechanical analysis and histological/gene expression analyses were conducted on separate samples, which made it difficult to analyze the structure-function relationship. Because the differences in some of the properties (ie, biomechanical and gene expression) were small and the animal-to-animal variation was relatively large, it was challenging to observe statistically significant differences in certain outcomes. Yet, another limitation of our study is the size of the animal model used and its physiological relevance to the human clinical condition.<sup>45</sup> A large animal model or perhaps a tendinopathy (collagenase injury) model would provide another translational method for analysis of the benefits of Doxy.

In summary, our findings suggest that the combination treatment of surgical repair and Doxy produces the greatest repair quality of Achilles tendons after an acute transection injury. The addition of Doxy to surgical repair of Achilles tendons may stimulate earlier remodeling of healing and should be further investigated. Interestingly, the administration of Doxy in the absence of surgical repair had little to no effect on the quality of tendon repair. The differential effect of Doxy on repaired and unrepaired tendons suggests that surgical repair is necessary for the enhanced effects of Doxy in Achilles tendon repair.

## ACKNOWLEDGMENT

The authors acknowledge Nina Kohn from the Department of Biostatistics for her expertise and help with the statistics of gene expression analysis.

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