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Doxycycline Improves Cage Activity, but not Exercised, Supraspinatus Tendon and Muscle in a Rat Model

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Abstract

The objective of this study was to investigate the effects of doxycycline, a broad-spectrum MMP inhibitor, on cage activity and exercised supraspinatus tendon and muscle using a Sprague-Dawley rat model of non-injurious exercise. Because exercise may alter muscle and tendon MMP activity and matrix turnover, we hypothesized that doxycycline would abolish the beneficial adaptations found with exercise but have no effect on cage activity muscle and tendon properties. Rats were divided into acute or chronic exercise (EX) or cage activity (CA) groups, and half of the rats received doxycycline orally. Animals in acute EX groups were euthanized 24 hours after a single bout of exercise (10 m/min, 1 hour) on a flat treadmill. Animals in chronic EX groups walked on a flat treadmill and were euthanized at 2 or 8 week time points. Assays included supraspinatus tendon mechanics and histology and muscle fiber morphologic and type analysis. Doxycycline improved tendon mechanical properties and collagen organization in chronic cage activity groups, which was not consistently evident in exercised groups. Combined with exercise, doxycycline decreased average muscle fiber cross-sectional area. Results of this study suggest that administration of doxycycline at pharmaceutical doses induces beneficial supraspinatus tendon adaptations without negatively affecting the muscle in cage activity animals, supporting the use of doxycycline to combat degenerative processes associated with underuse; however, when combined with exercise, doxycycline does not consistently produce the same beneficial adaptations in rat supraspinatus tendons and reduces muscle fiber cross-sectional area, suggesting that doxycycline is not advantageous when combined with activity.

Keywords

Doxycycline; Exercise; Matrix metalloproteinases; Rotator cuff; Tendon

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Conflict of Interest Statement

The authors have no conflicts of interest with this work.

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Introduction

Rotator cuff tendinopathy, primarily affecting the supraspinatus tendon, is a common clinical condition. Matrix metalloproteinases (MMPs) are enzymes that degrade extracellular matrix proteins such as collagen, the main structural component of tendon. MMPs have been implicated in the progression of tendon degeneration, which is supported by numerous studies that have found alterations in expression (Archambault, 2007; Attia, 2013; Sejersen, 2015). Although changes in MMP expression are characteristic of tendinopathy, non-injurious exercise also produces changes in tendon (e.g., Astill, 2017; De Mello Malheiro, 2009; Koskinen, 2004) and muscle (e.g., Rullman 2009; Rullman 2007; Yeghiazaryan 2012) MMP expression, consistent with matrix turnover and beneficial adaptations. Potentially, one distinguishing feature between maladaptation and beneficial adaptation to exercise is the role of MMPs, such as MMP activity levels, which MMPs are active, and whether they are balanced by their inhibitors.

MMP inhibition has been proposed to improve skeletal muscle and tendon healing (Davis, 2013). Doxycycline, in addition to being a commonly prescribed antibiotic, is an MMP inhibitor. The most potent and well-studied of the tetracyclines, doxycycline is widely accepted to be a broad-spectrum inhibitor of MMPs, including MMP-1, -2, -7, -8, -9, -12, and -13, through pathways independent of its antimicrobial effects (Pasternak, 2006). Studies investigating doxycycline on tendon healing are equivocal. Following acute detachment and repair of the rat supraspinatus tendon, doxycycline improved collagen organization and failure load at 2 but not 4 weeks (Bedi, 2010). After acute Achilles tendon injury in rats, doxycycline inhibited MMP activity, improving tendon histologic scores, collagen organization, and mechanical properties in two prior studies (Kessler, 2014; Nguyen, 2017), but decreased ultimate load in another study (Pasternak, 2006). Doxycycline also reduced the severity of skeletal muscle reperfusion injury (Roach, 2002). These prior studies were designed to investigate doxycycline effects on acute injuries, not chronic tissue adaptations. It is unknown how doxycycline affects muscle and tendon adaptations to exercise or homeostasis in sedentary tissues.

The objective of this study was to investigate the effects of doxycycline on cage activity and exercised supraspinatus tendon and muscle using our rat model of non-injurious exercise (Rooney, 2015A). With this model, we demonstrated distinct acute and chronic effects of exercise to the muscle and tendon and detected altered genes associated with matrix turnover (Rooney, 2015B; Rooney, 2017). We hypothesized that administration of doxycycline would abolish the beneficial adaptations of exercise but have no effect on cage activity muscle and tendon properties. Beneficial tissue adaptations often resulting from exercise include increased tendon mechanical properties (stiffness, modulus, maximum load, and maximum stress) and muscle hypertrophy (Rooney, 2015A; Rooney, 2017). Decreases in tendon mechanical properties or muscle fiber cross-sectional area in chronic cage-activity groups typically represent maladaptations or injury and in chronic exercised groups represent lack of beneficial adaptations to training.

Methods

Study Design and Treadmill Protocols

Following approval by the University of Pennsylvania's Institutional Animal Care and Use Committee, 165 adult, male Sprague-Dawley rats (400–450g at start of study) were divided into cage activity (CA) or exercise (EX) groups. These groups were further divided into one acute time point and two chronic time points. Half of the animals received doxycycline (DOX). The non-drug comparison groups are a subset of another study performed in the same time frame by the same personnel (Rooney, 2017). Animals in the DOX groups were administered an oil suspension of doxycycline hyclate (Wedgewood Pharmacy, Swedesboro, NJ, USA) orally every 24 hours at a dose of 10 mg/kg (standard veterinary clinical dose). This dose previously induced changes to the healing Achilles tendon via MMP inhibition (Kessler, 2014; Nguyen, 2017).

To investigate short-term effects of doxycycline on muscle and tendon responses to exercise, animals in the acute EX group underwent 2 weeks of graduated treadmill training, 72 hours of rest, and then a single, flat treadmill exercise session at 10 meters/minute for 1 hour (Rooney, 2015B; Rooney, 2017). These animals were euthanized 24 hours after their single bout of exercise (EX24). Animals in the DOX groups received doxycycline every 24 hours until euthanasia, beginning 24 hours prior to their exercise session (3 doses total, EX24DOX). A separate group of animals maintained normal cage activity (did not undergo treadmill training) and received 3 doses of doxycycline (1 dose every 24 hours) until euthanasia 1.5 hours after their last dose of doxycycline (CA24DOX). These animals were compared to a cage activity group that did not receive doxycycline or treadmill training (CA24, Figure 1).

To investigate the long-term effects of doxycycline on muscle and tendon adaptations to exercise, animals in chronic EX groups underwent 2 weeks of flat treadmill acclimation and then walked on a flat treadmill at 10 meters/minute, 1 hour per day, 5 days per week, for 2 or 8 weeks (EX2, EX8) (Rooney, 2017). These animals were euthanized 72 hours after their last exercise session to avoid potentially confounding acute effects of exercise (Rooney, 2015B; Rooney, 2017). Cage activity animals maintained normal cage activity for this duration (CA2, CA8) (Rooney, 2017). Animals in DOX groups received doxycycline every 24 hours, 7 days per week for the 2 or 8 week duration (EX2DOX, EX8DOX, CA2DOX, CA8DOX, Figure 1).

For exercise groups, inclusion was set to 75% completion of the total treadmill protocol. 90 out of 115 of the rats originally allotted to the exercise groups completed 75% of the exercise protocol; only these animals were included in the exercise group analysis. Animals that completed <10% of the treadmill protocol (did not complete 2 weeks of treadmill training and were removed before beginning exercise) were included in the CA groups. All rats were housed in an AALAC accredited facility (12/12 hour light/dark cycle, 20–26°C, and humidity 30–70%) and fed *ad libitum*. The cage activity group models a sedentary lifestyle (Martin, 2010; Spangenberg, 2005). Rats were euthanized with controlled flow-rate carbon dioxide. Shoulders from each rat were designated to muscle/tendon histology or tendon mechanics and prepared as previously described (Rooney, 2016). Tendon mechanical

testing was not performed on the CA24 or CA24DOX groups to reduce the number of animals since mechanical changes after only 24 hours were not expected. Given the study size and duration, it was not possible to randomize every animal from the vendor; however, animals were randomized within groups. Further, although the investigators were not formally blinded, the investigators performing the assays were not aware of which groups the animals belonged at time of testing.

Tendon Mechanical Testing

A random permutation in MATLAB randomized tendon mechanical testing order. Tendon cross-sectional area was measured with a custom laser device (Peltz, 2009), and Verhoeff's stain lines placed at the insertion site and 4 mm proximally were used for optical strain measurements. Tendons underwent a previously described testing protocol (Rooney, 2016). Analyzed data included a stress-relaxation to 8% strain held for 300s, a frequency sweep (0.1, 1, 2, 10 Hz) at 8% strain of 10 sine cycles with amplitude of 0.125% strain, and a ramp to failure at 0.3%/s.

Stiffness of the whole tendon-bone construct was calculated as the linear slope of the ramp to failure load-displacement curve. Local 2D Lagrangian strain (measured by optically tracking the stain lines) was used to calculate local elastic modulus. Percent relaxation was calculated as the percent change in load after 300 seconds. The dynamic modulus ($|E^*|$) and tangent of the phase angle between the stress and strain ($\tan(\delta)$) were calculated at each frequency of the frequency sweep.

Tendon Histology

Formalin-fixed, decalcified, paraffin-embedded bone-tendon-muscle units were sectioned (7 μ m), stained with hematoxylin and eosin, and analyzed as previously described (Rooney, 2016). One tendon midsubstance section per sample was imaged at 200x magnification and used for traditional (acute and chronic groups) and polarized (chronic groups only) light microscopy. Commercial software (Bioquant Osteo II, Nashville, TN, USA) quantified cell density (cells/mm²) and cell shape (aspect ratio 0–1 with 1 being a circle). Custom MATLAB software analyzed the polarized light images and calculated the circular standard deviation, a measure of the spread of collagen fiber alignment (Gimbel, 2004). Group sample sizes are provided in the corresponding figure legends.

Muscle Histology

Muscle specimens were cryosectioned (10 μ m) transversely to the fibers (Rooney, 2016). Morphological analysis was performed using laminin and DAPI immunofluorescence. From each sample, six images were acquired at 100x magnification (>450 fibers per specimen). The percent of centrally nucleated fibers (CNF) was calculated as the number of CNFs divided by the total number of fibers measured. The total average fiber cross-sectional area was analyzed with a MATLAB application (Smith, 2014). Fiber type analysis, as previously described (Rooney, 2016), was performed using immunofluorescence by simultaneously staining with anti-myosin heavy chain-I (MyHC-I) BA-DF, MyHC-IIa SC-71, and MyHC-IIb BF-F3 antibodies (Schiaffino, 1989) (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, IA, USA), and anti-laminin antibody (Sigma-Aldrich, St.

Louis, MO, USA). MyHC-IIx fibers were identified as the unstained fibers. Because the superficial and deep layers of the rat supraspinatus muscle differ (Barton, 2005; Rooney, 2016), these regions were analyzed separately for muscle fiber type distribution and cross-sectional area (Smith, 2014).

Statistics

Statistical analysis was performed based on our hypotheses of the effects of doxycycline in cage activity and (separately) exercised groups. A pre-study power analysis using data from a previous study (Kessler, 2014) that administered the same dose of doxycycline and investigated rat Achilles tendon healing estimated a required sample size of 10 specimens per group to detect a doxycycline effect size of 1.2 on tendon stiffness. The primary outcome measures used to determine whether our hypotheses were supported included tendon mechanical properties and muscle fiber cross-sectional area. Specifically, if our hypothesis were supported in cage activity groups, we expected no change in tendon stiffness, modulus, maximum load, or maximum stress and no change in muscle fiber cross-sectional area with administration of doxycycline at either 2 or 8 week chronic time points. If our hypothesis were supported in the chronic exercise groups, we expected decreased tendon stiffness, modulus, maximum load, and maximum stress, particularly in the 2 week group, and decreased muscle fiber cross-sectional area. Since we previously reported more adaptations to exercise at 2 weeks than 8 weeks (Rooney, 2017), the 2 week time point was of particular interest. In addition, we previously showed that 24 hours after a single bout of exercise, the supraspinatus tendon exhibits increased cross-sectional area and mild trends toward decreased modulus, maximum stress, and maximum load (Rooney, 2017). Therefore, if our hypothesis were supported acutely, we would expect decreased tendon cross-sectional area and increased modulus, maximum stress, and maximum load. To determine the effects of doxycycline on muscle and tendon adaptations, t-tests compared DOX and non-drug groups separately for CA and EX at each time point. Comparisons between time points are inappropriate due to the significant animal growth that occurs. Since our hypotheses were specific to the effects of doxycycline, and we have previously reported on the effects of exercise compared to cage activity (Rooney, 2017), we did not compare exercise and cage activity groups. All p-values, uncorrected for multiple comparisons, are reported. Significance was set to $p = 0.05$. Figures show mean and standard deviation.

Results

Tendon Mechanics

Combined with an acute bout of exercise, doxycycline (EX24DOX compared to EX24) decreased tendon cross-sectional area and increased tendon modulus, maximum stress (Figure 2), and dynamic modulus (Figure 3). Viscoelastic properties (percent relaxation- Figure 2D and $\tan(\delta)$ - Figure 4) of tendons following a single bout of exercise were not altered by doxycycline.

Combined with chronic exercise, doxycycline decreased tendon cross-sectional area in the 2 week group and increased modulus and maximum stress for the 8 week group (Figure 2). Doxycycline combined with exercise decreased maximum load at 2 weeks and decreased

stiffness at 8 weeks (Figure 2). Doxycycline also increased dynamic modulus at all frequencies for the 2 week exercise time point but not the 8 week exercise time point (Figure 3). Viscoelastic properties were also altered in the chronic exercise groups combined with doxycycline. Specifically, combined with 2 weeks of exercise, doxycycline increased percent relaxation (Figure 2). For both the 2 and 8 week exercise groups, doxycycline increased $\tan(\delta)$ at the lower frequencies (0.1 and 1 Hz, Figure 4).

Combined with cage activity, doxycycline significantly increased tendon modulus, maximum stress, and stiffness (Figure 2) and dynamic modulus at all frequencies (Figure 3) at both 2 and 8 weeks. Maximum load increased following 2 weeks of doxycycline administration in the cage activity group. Percent relaxation decreased after 2 weeks of cage activity combined with doxycycline but increased after 8 weeks (Figure 2). Doxycycline increased $\tan(\delta)$ for the 8 week cage activity group at the lowest frequency (0.1 Hz) but decreased $\tan(\delta)$ for the 2 week cage activity group at the two highest frequencies (2 Hz, 10 Hz, Figure 4).

Tendon Histology

Acute administration of doxycycline did not significantly affect cell density or cell shape for cage activity or exercised groups (Figure 5). At 8 weeks, doxycycline decreased cell density in cage activity and exercise groups. Doxycycline administration resulted in rounder cells in the cage activity groups at 2 and 8 weeks and the exercise group at 8 weeks (Figure 5). Tendon collagen organization increased (decreased circular standard deviation) in the cage activity group with 8 weeks of doxycycline (Figure 6). The mild changes in cell density and cell shape and highly organized collagen contrast those seen with injury.

Muscle Histology

For all groups, centrally nucleated fibers comprised on average less than 1% of muscle fibers, and doxycycline had no effect (Table 1, Figure 7B). Doxycycline decreased the average total muscle fiber cross-sectional area in the acute exercise and 8 week exercise groups but did not affect cage activity groups (Figure 7).

We did not detect any statistically significant shifts in muscle fiber type distribution due to doxycycline in cage activity or exercise groups (Table 2). Muscle fiber type-specific crosssectional area changes due to doxycycline were evident only in the chronic exercise groups (Table 3). Following 2 weeks of chronic exercise, doxycycline reduced MyHC-IIb and MyHCIIx fiber cross-sectional area in the superficial region of the supraspinatus (Table 3). In the deep region of the muscle, MyHC-I fiber cross-sectional area increased and MyHC-IIb cross-sectional area decreased in the 2 week exercise group administered doxycycline. After 8 weeks of exercise, doxycycline decreased MyHC-IIb fiber cross-sectional area. Doxycycline did not affect muscle fiber cross-sectional area in cage activity groups.

Discussion

This study is the first to show that doxycycline impacts cage activity and exercised supraspinatus muscle and tendon. Contrary to our hypothesis, doxycycline significantly

affected cage activity supraspinatus tendon properties. Surprisingly, in the cage activity groups, chronic doxycycline administration increased tendon mechanics (stiffness, modulus, max load, max stress, and dynamic modulus) and organization. Increases in these mechanical properties are similar to what we previously reported as beneficial tissue adaptations to exercise (Rooney, 2017) and align with our primary outcome measures. Chronic administration of doxycycline appears beneficial for cage activity tendon and does not appear to impact cage activity muscle, which may be due to alterations in MMP activity levels in the tendon as a result of underuse.

These same increases in tendon mechanical properties were not always present in chronically exercised tendons. In fact, doxycycline combined with chronic exercise decreased tendon stiffness at 8 weeks and maximum load at 2 weeks and also decreased muscle fiber crosssectional area. Doxycycline does not appear to benefit chronically exercised supraspinatus tendon and muscle.

Acutely, doxycycline combined with a single bout of exercise reduced tendon cross-sectional area and increased modulus and maximum stress, bringing these properties closer to previously measured baseline levels (Rooney, 2017). We previously showed that 24 hours following a single bout of exercise, tendon cross-sectional area increases, and there are mild trends towards reduced mechanical properties, which may lead to enhanced tendon mechanical properties over time (Rooney, 2017). In the current study, we showed that doxycycline negates some of these mild, acute responses of the tendon to exercise and consequently may lead to diminished beneficial effects of exercise over time.

These beneficial adaptations of tendons from cage activity animals receiving doxycycline, an MMP-inhibitor, suggest that MMP activity is higher in cage activity than exercised tendons. Supporting this, we previously showed that supraspinatus tendons from rats that maintained cage activity had increased generic MMP activity after 8 weeks compared to rats that underwent chronic exercise for the same duration (Rooney, 2017). In addition, the cage activity group demonstrated increased generic MMP activity compared to 12, 24, and 48 hours following a single bout of exercise (Rooney, 2017). The rats from this previous study (Rooney, 2017), which was performed in the same time frame by the same personnel, served as the control (non-drug administered) animals in the current study. These findings suggest that cage activity supraspinatus tendons, similar to underused tendons, express heightened matrix degradation facilitated by MMPs. A previous *in vitro* study showed increased MMP-13 mRNA expression with stress-deprived tendons, and administration of ilomastat (broad-spectrum MMP inhibitor) recovered the gene expression (Gardner, 2008). Similarly, the MMP inhibitors doxycycline and ilomastat administered to rat tail tendons undergoing one week of *in vitro* stress deprivation prevented the detrimental loss of mechanical properties (Arnoczky, 2007). The results of the current study support the findings of these previous studies, suggesting that tendon disuse or underuse heightens MMP activity and consequently increases degenerative, catabolic processes. Doxycycline, a known MMP inhibitor, may prevent these detrimental losses associated with underuse. Exercise can also benefit by decreasing MMP activity, thereby enhancing tendon mechanical properties compared to cage activity (Rooney, 2017).

Muscle from exercised animals receiving doxycycline had decreased total fiber cross-sectional area, which was not evident in cage activity groups. Exercise combined with doxycycline for 2 weeks increased MyHC-I muscle fiber cross-sectional area in the deep region of the supraspinatus muscle and decreased MyHC-IIx (superficial region) and MyHC-IIb (superficial and deep regions) muscle fiber cross-sectional area, suggesting that the fatigueresistant type I fibers may be playing a more important role than the fatigable type IIb and IIx fibers; however, by 8 weeks of exercise combined with doxycycline, only a significant decrease in type IIb fiber cross-sectional area was detected. Overall, changes in muscle fiber cross-sectional area were evident in the exercise, but not cage activity, groups.

Several studies have reported altered MMP expression with tendinopathy. Beneficial adaptations to exercise result in a synthesis-to-degradation ratio that favors of synthesis, whereas maladaptations leading to tendinopathy result in degradation overpowering synthesis. The results of this study, combined with findings of previous studies, support that even cage activity can shift tendons towards degradation, and administration of doxycycline can recover some of these lost properties. Results also support that exercise, previously shown to reduce MMP activity levels (Rooney, 2017), combined with doxycycline, a known MMP inhibitor, causes some concerning tissue adaptations: decreased muscle hypertrophy, reduced tendon stiffness at 8 weeks, and reduced tendon maximum force at 2 weeks. These chronic consequences in the tendon could be a result of the impact doxycycline has on the acute tendon response following a single bout of exercise. Additionally, the chronic effects may be due to an interaction between doxycycline and exercise.

This study is not without limitations. First, this study may not have been sufficiently powered to detect small differences between groups; however, we did detect several statistically significant changes with administration of doxycycline. Most importantly, we identified increased tendon stiffness, modulus, and maximum stress after 2 and 8 weeks of cage activity and increased maximum load after 2 weeks. Furthermore, doxycycline did not decrease muscle fiber cross-sectional area. These were the key outcome measures defined in this study. Taken together, these changes suggest that doxycycline benefited the supraspinatus tendon in cage activity groups. Additionally, muscle function was not measured; it is unknown whether the reported muscle fiber type-specific changes result in functional differences. In this study, doxycycline was administered orally, as is normally done clinically. Although we did not confirm that doxycycline administration decreased local MMP activity, the dose we used (10mg/kg daily) has previously been shown to induce clinically relevant serum levels of the drug, decrease MMP activity in a rat Achilles transection after 4 weeks, and improve tendon healing (Kessler, 2014; Nguyen, 2017), providing substantial support for this dose and its efficacy in reducing MMP activity in tendon. Timing of drug administration could also impact the findings in this study; both cage activity and exercised DOX groups were consistently dosed every 24 hours in the morning, and it is unknown how results would differ if drug administration were timed differently. Little information is available on how exercise impacts doxycycline pharmacokinetics. Ylitalo et. al. (1977) reported increased doxycycline serum concentration for up to 24 hours and delayed doxycycline excretion with exercise compared to bed rest in young, healthy adults. These prior findings are difficult to generalize to a rat model of sustained doxycycline administration and mild exercise but do support the clinical importance of

investigating the effects of doxycycline when combined with physical activity. Importantly, we did detect significant differences with administration of doxycycline combined with exercise, supporting that the drug was locally present and effective in the tissue. In addition, this study used a single exercise protocol, and it is unknown how results would change with increased exercise intensity; however, we have previously shown that this protocol produces significant adaptations to the rat shoulder without inducing supraspinatus tendon injury, indicating that it is a physiologically relevant model of non-injurious exercise (Rooney, 2015A; Rooney, 2015B; Rooney, 2017). Future studies can explore the effects of doxycycline in overuse and stressdeprived conditions. Finally, due to risks associated with unnecessary antibiotic use, we do not recommend that healthy, sedentary individuals be prescribed doxycycline; however, these results support future research on chemically modified tetracyclines or other MMP inhibitors (Pasternak, 2009).

In this study, we detected several effects of doxycycline on cage activity tissues that mimicked the beneficial effects of exercise: enhanced tendon mechanical properties and consistently healthy tendon histology with improved collagen organization. These improved tendon properties occurred in response to doxycycline without reducing the muscle fiber crosssectional area or increasing the number of centrally nucleated fibers. Future studies should further explore these exercise mimetic properties of doxycycline and other MMP inhibitors on sedentary tissues. In conclusion, results of this study suggest that administration of doxycycline at pharmaceutical doses induces beneficial supraspinatus tendon adaptations without negatively affecting the muscle in cage activity animals, supporting the use of an MMP inhibitor to combat degenerative processes associated with underuse; however, when combined with exercise, doxycycline does not consistently produce the same beneficial adaptations in rat supraspinatus tendons and reduces muscle fiber cross-sectional area, suggesting that doxycycline is not advantageous when combined with activity.

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Figure 1. Acute (A) and Chronic (B) Study Designs.

A) Rats in the acute cage activity group maintained normal cage activity, received 3 doses of doxycycline, were euthanized 1.5 hours after their last dose of the drug (CA24DOX), and were compared to a group that did not receive doxycycline (CA24). Rats in acute exercise groups underwent 2 weeks of treadmill training followed by 72 hours of rest. These rats were euthanized 24 hours after completion of a single bout of exercise (EX24). These animals were compared to a group that received doxycycline beginning 1 day before their exercise session and continuing until euthanasia (EX24DOX). **B)** For analysis of chronic effects, cage activity groups maintained normal cage activity for an early time point (CA2, CA2DOX) or a later time point (CA8, CA8DOX). Rats in chronic exercise groups underwent 2 weeks of treadmill training followed by 2 or 8 weeks of the exercise protocol (EX2, EX2DOX, EX8, EX8DOX). DOX groups received doxycycline during this 2 or 8 week period. Assays were performed on supraspinatus: M- tendon mechanics, TH- tendon histology, MH- muscle histology, N- number of animals allotted to that group

Figure 3. Dynamic Modulus.

Administration of doxycycline combined with a single bout of exercise increased tendon dynamic modulus after 24 hours. Chronically, dynamic modulus increased at the 2 week time point for both cage activity and exercise groups but increased in only the cage activity group at the 8 week time point. Shaded bars indicate the DOX groups at each time point. Separate t-tests were performed at each activity level/time point to compare DOX-treated and control groups. Mean and standard deviation shown. * indicates $p < 0.05$. Sample sizes: EX24 $n=11$, EX24DOX $n=13$, CA2 $n=17$, CA2DOX $n=13$, EX2 $n=13$, EX2DOX $n=13$, CA8 $n=12$, CA8DOX $n=10$, EX8 $n=11$, EX8DOX $n=14$

Figure 4. Tan(δ).

Administration of doxycycline combined with a single bout of exercise had no effect on tan(δ). In chronic cage activity groups, tan(δ) increased with doxycycline administration at the 8 week time point at 0.1 Hz and decreased at the 2 week time point at 2 and 10 Hz. In contrast, combined with chronic exercise, doxycycline increased tan(δ) at both 2 and 8 weeks at 0.1 and 1 Hz and had no effect at 2 and 10 Hz. Shaded bars indicate the DOX groups at each time point. Separate t-tests were performed at each activity level/time point to compare DOX-treated and control groups. Mean and standard deviation shown. * indicates $p < 0.05$. Sample sizes: EX24 $n=11$, EX24DOX $n=15$, CA2 $n=17$, CA2DOX $n=13$, EX2 $n=12$, EX2DOX $n=15$, CA8 $n=11$, CA8DOX $n=11$, EX8 $n=12$, EX8DOX $n=14$

Figure 5. Tendon Histology.

Administration of doxycycline had no acute effects on cell density (**A**) or cell shape (**B**). Chronic administration led to fewer and rounder cells at 8 weeks for both cage activity and exercise groups and rounder cells in the cage activity group at 2 weeks. Shaded bars indicate the DOX groups at each time point. Representative tendon histology images are shown in panel **C**. Scale bar is 50 microns. Separate t-tests were performed at each activity level/time point to compare DOX-treated and control groups. Mean and standard deviation shown. * indicates $p < 0.05$. Sample sizes: EX24 $n=7$, EX24DOX $n=7$, CA2 $n=9$, CA2DOX $n=7$, EX2 $n=8$, EX2DOX $n=7$, CA8 $n=6$, CA8DOX $n=6$, EX8 $n=8$, EX8DOX $n=7$

Figure 6. Tendon Collagen Organization.

After 8 weeks of cage activity, doxycycline increased collagen organization. Shaded bars indicate the DOX groups at each time point. Separate t-tests were performed at each activity level/time point to compare DOX-treated and control groups. Mean and standard deviation shown. * indicates $p < 0.05$. Sample sizes: CA2 $n=8$, CA2DOX $n=7$, EX2 $n=8$, EX2DOX $n=7$, CA8 $n=6$, CA8DOX $n=6$, EX8 $n=8$, EX8DOX $n=7$

Figure 7. Average Muscle Fiber Cross-Sectional Area.

A) Administration of doxycycline decreased average muscle fiber cross-sectional area in the exercise, but not cage activity, groups acutely and after 8 weeks. Shaded bars indicate the DOX groups at each time point. Separate t-tests were performed at each activity level/time point to compare DOX-treated and control groups. Mean and standard deviation shown. * indicates $p < 0.05$. Sample sizes: CA24 $n=8$, CA24DOX $n=8$, EX24 $n=7$, EX24DOX $n=7$, CA2 $n=9$, CA2DOX $n=7$, EX2 $n=8$, EX2DOX $n=7$, CA8 $n=6$, CA8DOX $n=6$, EX8 $n=8$, EX8DOX $n=7$ **B)** Representative muscle histology images stained for laminin (green) and cell nuclei with DAPI (blue). This EX8 sample had an average fiber size of $4105 \mu\text{m}^2$ and this EX8DOX sample had an average fiber size of $3513 \mu\text{m}^2$. Minimal centrally nucleated fibers were seen. Scale bar is $100 \mu\text{m}$.

Table 1.**Centrally Nucleated Muscle Fibers (CNF), %.**

For all groups, the average percent of centrally nucleated fibers was below 1%. Values are mean $\pm$ standard deviation. Separate t-tests were performed at each activity level/time point to compare DOX-treated and control groups. Doxycycline had no effect ($p > 0.05$ for all comparisons) on CNF. N = sample size

Group	No drug		Doxycycline		P-value
	% CNF	N	% CNF	N	
CA24	0.31 $\pm$ 0.06	7	0.34 $\pm$ 0.30	8	0.4
EX24	0.49 $\pm$ 0.40	7	0.36 $\pm$ 0.20	7	0.2
CA2	0.32 $\pm$ 0.22	8	0.29 $\pm$ 0.25	7	0.4
EX2	0.34 $\pm$ 0.08	7	0.39 $\pm$ 0.24	7	0.3
CA8	0.40 $\pm$ 0.40	6	0.55 $\pm$ 0.23	6	0.2
EX8	0.61 $\pm$ 0.25	7	0.59 $\pm$ 0.33	7	0.5

Muscle Fiber Type Distribution, %.

Table 2.

Superficial and deep regions of the supraspinatus muscle were analyzed separately for percent fiber type. The superficial region had no MyHC-I staining. No significant impact of doxycycline on fiber type distribution was detected. Values are mean $\pm$ standard deviation. Separate t-tests were performed at each activity level/time point to compare DOX-treated and control groups. N = sample size

Group	N	Superficial			N	Deep			
		MyHC-Ia	MyHC-IIb	MyHC-IIx		MyHC-I	MyHC-IIa	MyHC-IIb	MyHC-IIx
CA2	9	9 $\pm$ 3	66 $\pm$ 6	25 $\pm$ 7	7	13 $\pm$ 2	29 $\pm$ 5	26 $\pm$ 7	32 $\pm$ 3
CA2DOX	6	11 $\pm$ 3	67 $\pm$ 3	22 $\pm$ 2	6	14 $\pm$ 2	35 $\pm$ 9	21 $\pm$ 8	29 $\pm$ 5
P-value		0.1	0.3	0.1		0.2	0.07	0.1	0.1
EX2	8	16 $\pm$ 6	57 $\pm$ 10	28 $\pm$ 7	7	13 $\pm$ 3	37 $\pm$ 6	21 $\pm$ 8	29 $\pm$ 5
EX2DOX	7	14 $\pm$ 6	49 $\pm$ 12	38 $\pm$ 17	5	14 $\pm$ 3	40 $\pm$ 5	16 $\pm$ 3	31 $\pm$ 5
P-value		0.2	0.1	0.07		0.3	0.3	0.1	0.3
CA8	6	11 $\pm$ 5	60 $\pm$ 8	28 $\pm$ 4	6	16 $\pm$ 3	30 $\pm$ 3	18 $\pm$ 5	36 $\pm$ 3
CA8DOX	6	10 $\pm$ 7	57 $\pm$ 8	33 $\pm$ 7	6	15 $\pm$ 5	36 $\pm$ 9	16 $\pm$ 8	33 $\pm$ 4
P-value		0.3	0.3	0.09		0.4	0.08	0.3	0.09
EX8	8	18 $\pm$ 6	51 $\pm$ 8	31 $\pm$ 5	7	13 $\pm$ 4	42 $\pm$ 6	16 $\pm$ 7	29 $\pm$ 2
EX8DOX	6	15 $\pm$ 2	54 $\pm$ 6	31 $\pm$ 6	6	12 $\pm$ 1	41 $\pm$ 6	16 $\pm$ 7	31 $\pm$ 3
P-value		0.2	0.2	0.4		0.3	0.4	0.5	0.1

Table 3.

Muscle Fiber Type Cross-Sectional Area, μm^2 .

Superficial and deep regions of the supraspinatus muscle were analyzed separately for fiber type cross-sectional area. The superficial region had no MyHC-I staining. Only the exercised groups showed a response to doxycycline administration. Values are mean $\pm$ standard deviation. Separate t-tests were performed at each activity level/time point to compare DOX-treated and control groups.

Group	N	Superficial			N	Deep			
		MyHC-IIa	MyHC-IIb	MyHC-IIx		MyHC-I	MyHC-IIa	MyHC-IIb	MyHC-IIx
CA2	8	1455 $\pm$ 198	3614 $\pm$ 510	2174 $\pm$ 309	8	1764 $\pm$ 242	2151 $\pm$ 204	4048 $\pm$ 365	3099 $\pm$ 311
CA2DOX	7	1491 $\pm$ 262	3682 $\pm$ 334	2118 $\pm$ 239	6	1830 $\pm$ 324	2190 $\pm$ 321	3946 $\pm$ 332	2969 $\pm$ 411
P-value		0.4	0.4	0.4		0.3	0.4	0.3	0.3
EX2	8	1611 $\pm$ 166	3848 $\pm$ 547	2447 $\pm$ 344	7	1769 $\pm$ 190	2220 $\pm$ 196	4056 $\pm$ 617	3053 $\pm$ 295
EX2DOX	7	1462 $\pm$ 190	3348 $\pm$ 240	2103 $\pm$ 248	5	1994 $\pm$ 192	2281 $\pm$ 355	3140 $\pm$ 518	2696 $\pm$ 533
P-value		0.06	*0.02	*0.02		*0.04	0.4	*0.01	0.08
CA8	6	1845 $\pm$ 180	4225 $\pm$ 485	2761 $\pm$ 438	6	2069 $\pm$ 333	2783 $\pm$ 481	4552 $\pm$ 658	3918 $\pm$ 612
CA8DOX	6	1636 $\pm$ 289	4270 $\pm$ 474	2562 $\pm$ 566	6	2105 $\pm$ 442	2600 $\pm$ 531	4441 $\pm$ 512	3518 $\pm$ 588
P-value		0.08	0.4	0.3		0.4	0.3	0.4	0.1
EX8	8	1890 $\pm$ 304	4669 $\pm$ 415	3001 $\pm$ 614	7	2083 $\pm$ 357	2720 $\pm$ 291	5088 $\pm$ 349	3714 $\pm$ 399
EX8DOX	6	1714 $\pm$ 67	4555 $\pm$ 674	2622 $\pm$ 372	7	1930 $\pm$ 252	2550 $\pm$ 144	4654 $\pm$ 391	3616 $\pm$ 306
P-value		0.1	0.4	0.1		0.2	0.09	*0.02	0.3

* indicates p 0.05. N = sample size