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Effect of simvastatin on rat supraspinatus tendon mechanical and histological properties in a diet-induced hypercholesterolemia model

Jennica J Tucker, MSE and Louis J. Soslowsky, PhD

McKay Orthopaedic Research Laboratory, Philadelphia, PA

Abstract

Hypercholesterolemia is a common condition and is a risk factor for tendon rupture, specifically in the supraspinatus tendon. In the clinic, statins are commonly prescribed to lower cholesterol, but little information is available examining the effect of statin treatment on the musculoskeletal system. Therefore, the objective of this study was to determine the biomechanical and histological effects of statin treatment in a diet-induced hypercholesterolemia model. We hypothesized that hypercholesterolemic rats treated with statins would have improved tendon biomechanical and histological properties compared to hypercholesterolemic rats not receiving daily statin treatment. Thirty adult male Sprague-Dawley rats ate either high-cholesterol (HC) diet (n=20) or normal chow (CTL, n=10). After six months, a subset of HC rats began daily oral simvastatin dosing (HC +S) at 20mg/kg. All rats were sacrificed after a total of nine months (three months of statin treatment) and evaluated for histology and mechanics. For mechanics, at the insertion region, HC +S rats had increased tendon cross-sectional area decreased and modulus. Modulus values approached CTL baseline values. No differences were noted in mechanical properties at the midsubstance. For histology, no differences were noted in the insertion region. In the midsubstance region, HC+S group had more spindle shaped cells, approaching normal CTL baseline values. Our results suggest that three months of simvastatin treatment in a diet-induced hypercholesterolemia rat model improves tendon mechanical and histological properties. Additionally, this data suggests that simvastatin use does not negatively affect tendon mechanical properties and might help to reduce the risk of tendon rupture.

Keywords

statin; simvastatin; hypercholesterolemia; supraspinatus; animal model

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Corresponding author: Louis J. Soslowsky, PhD, McKay Orthopaedic Research Laboratory, 424 Stemmler Hall, 3450 Hamilton Walk, Philadelphia, PA 19104-6081, Phone: 215-898-8653, Fax: 215-573-2133, soslowsk@upenn.edu.

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INTRODUCTION

Musculoskeletal disorders are highly prevalent in the United States representing yearly estimated direct and indirect costs of \$950 billion¹. In particular, rotator cuff tendon tears are particularly common, affecting up to 50% of Americans over 50 years old¹. Additionally, hypercholesterolemia is a common condition in which more than 27% of Americans over 20 years old have been diagnosed². Hypercholesterolemia is known to increase serum total cholesterol (TC), triglycerides (TG), and low-density lipoprotein (LDL), which negatively affect cardiovascular health³. While it is also a risk factor for tendon rupture, specifically in the supraspinatus and Achilles tendons⁴, high cholesterol is often diagnosed when a patient presents to an orthopaedic surgeon. A previous study found that patients presenting to orthopaedic surgeons with Achilles tendon ruptures reported 83% of patients had high cholesterol, and more than three quarters were undiagnosed³. Another study found that patients with often unknown familial hypercholesterolemia tended to present with Achilles tendon disorders more than 20 years prior to discovering they had high cholesterol⁵.

Diet-induced hypercholesterolemia is commonly studied in animal models. Specifically, previous studies in our laboratory have shown alterations in tendon properties in the rat^{6, 7}, mouse⁸, and non-human primate⁶. Of particular interest, in the rat model, we found increased stiffness and elastic modulus in the supraspinatus tendon in response to animals eating a high-cholesterol diet for six months⁶. In an otherwise healthy tendon, altered mechanical properties might predispose the tendon to chronic tendinopathy and eventual rupture.

Statins are drugs which lower cholesterol levels via blocking the enzyme 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase, which plays a role in cholesterol production in the liver^{9, 10}. In the clinic, physicians commonly prescribe statins to patients presenting with hypercholesterolemia, as they not only lower serum cholesterol levels, but also have been found to reduce the risk of cardiovascular disease in hypercholesterolemic patients¹¹.

At present, little information is available examining the effect of statin treatment for hypercholesterolemia in the musculoskeletal system, where most of the available literature examines the effect of statins in a normocholesterolemic state. One study reported decreased biomechanical strength of Achilles tendons in rats treated with statins, which might lead to increased risk of Achilles tendon rupture¹². Another study examining ruptured rat rotator cuff tendons found simvastatin treatment produced a reduction in fibrosis and muscle weakness¹³, suggesting statins have a positive effect on healing tissue. Given this conflicting information, there is a need to examine the effects of statins in a clinically relevant and highly controlled diet induced hypercholesterolemia rat model.

Therefore, the objective of this study was to determine the biomechanical and histological effects of statin treatment in a diet-induced hypercholesterolemia model^{6, 14}. Specifically, we examined these effects using an established rat rotator cuff model, which has been published extensively by us and others due to the anatomical similarities between rat and human shoulders¹⁵⁻¹⁸. We hypothesized that hypercholesterolemic rats treated with

simvastatin would have improved tendon biomechanical and histological properties compared to hypercholesterolemic rats not receiving daily simvastatin treatment.

METHODS

Study design

Thirty adult male Sprague-Dawley rats (400–450g) were used in this University of Pennsylvania Institutional Animal Care and Use Committee approved study. Rats were housed in pairs in a conventional facility with 12 hour light/dark cycles. At the beginning of the study, rats were randomly stratified into three groups of ten. To induce hypercholesterolemia (HC), rats were fed a high cholesterol diet (n=20) for six months. The HC diet is a modification of standard rat chow, consisting of 4% cholesterol and 1% sodium cholate (Research Diets, Inc, New Brunswick, NJ, USA, catalog #C12595) which has been shown to increase TC values in Sprague-Dawley rats^{6; 14; 19}. Age-matched control rats (CTL group, n=10) ate standard rat chow for six months. All rats were allowed food and water ad libitum and were weighed weekly throughout the study. After the initial six month treatment, a subset of the HC rats (n=10) were orally dosed with simvastatin (20mg/kg) daily for three months while continuing with the HC diet (HC+S group). All rats were sacrificed after a total of 9 months (fig. 1). Blood was collected from all rats at 6 months to confirm high-cholesterol in the HC groups and again at the time of sacrifice to measure TC, HDL, and TG. Additionally, the ratio of TC to HDL (TC/HDL) was calculated as a clinically relevant parameter. Immediately following sacrifice, right supraspinatus muscle-tendon-humerus units were dissected and fixed in formalin for histological analysis. Left limbs were then frozen at -20°C and later thawed for mechanical testing.

Simvastatin administration

Pharmaceutical-grade simvastatin (80mg tablets, Cadila Pharmaceuticals, Ahmedabad, India) was ground with a mortar and pestle and mixed with a honey-water mixture to increase palatability. Simvastatin suspension (30mg/ml) was mixed fresh weekly. Rats received 20mg/kg simvastatin via an oral dosing procedure previously employed in our laboratory^{20; 21}.

Tendon mechanical testing

At the time of testing, left limbs were thawed and supraspinatus-humerus tendon-bone units were dissected and cleaned of excess soft tissue under a stereomicroscope. Stain lines were placed along the length of the tendon for optical strain measurement. Cross sectional area was measured using a custom laser device²². The humerus was embedded in polymethylmethacrylate (PMMA) and held in a custom fixture and the tendon was affixed between fine grit sand paper using cyanoacrylate and then fixed in a custom grip. Samples were immersed in a 37°C PBS bath and subjected to a mechanical testing protocol consisting of a preload to 0.08 N, ten cycles of preconditioning (0.1–0.5 N at 1% strain/s), a stress relaxation to 5% strain (5%/s) followed by a 600s hold, and finally a ramp to failure at 0.3%/s. Stress was calculated as force divided by cross sectional area and 2D Lagrangian strain was determined from stain line displacements measured from images taken throughout the test using custom tracking software^{23; 24}. Mechanical properties were calculated for

both the insertion region and the midsubstance, with the insertion region defined as the distal 2 mm of the tendon (closest to the point of bony attachment) and the midsubstance region defined as the 2 mm proximal to the insertion region (toward the myotendinous junction).

Histology

Histological analysis was performed to assess cell morphology and collagen fiber organization at the insertion and midsubstance regions of the supraspinatus tendon. At the time of sacrifice, supraspinatus-humerus units were immediately dissected, fixed in formalin, and processed using standard paraffin techniques. 7µm sections were stained with hematoxylin and eosin (H&E) to assess cell shape, cellularity, and collagen fiber orientation. Cell shape and cellularity were evaluated by three blinded graders using a semi-quantitative method²⁵. Circular standard deviation (CSD) of collagen fibers was determined by images taken with a polarizing microscope and analysis with custom software as described previously^{24; 26; 27}.

Statistical Analysis

An *a priori* power analysis was performed based on data from previous similar studies to determine sufficient sample sizes. All comparisons were made between the HC and HC+S groups only (except for serum lipid panels at 6 months to confirm HC and weight comparisons over time). Mechanical parameters and CSD comparisons were made using student's t-tests with significance set at $p = 0.05$. Cell shape and cellularity comparisons were made using non-parametric Mann-Whitney tests with significance set at $p = 0.05$. Control animals served as age-matched comparisons and therefore statistical comparisons for *ex vivo* assays were not made between CTL and HC or HC+S groups as those comparisons were not required for our research question.

RESULTS

Serum lipid analysis (table 1) demonstrated that after six months, the HC diet significantly increased TC, HDL, and TC/HDL, and significantly decreased TG in the animals being fed the HC diet. After three months of simvastatin treatment, animals in the HC+S group had significantly decreased HDL and trended toward decreased TC compared to the HC group. No significant differences were noted in TC/HDL or TG. Animals in the HC and HC+S groups were significantly lighter than the CTL rats after introduction to the HC diet throughout the duration of the study (fig. 2). No significant differences in weight were noted between the HC and HC+S groups at all time points.

Importantly, there were no statistically significant differences in body weight between the HC and the HC+S groups at any time measurements were taken (weekly) throughout the study, as differences in body weight might affect tissue level outcome comparisons between groups.

Tendon mechanical properties

In the insertion region, the HC+S group had significantly increased cross-sectional area (18%) and significantly decreased elastic modulus (42%) compared to the HC group (fig. 3a,

b respectively). In the midsubstance region, no differences were observed in cross-sectional area or elastic modulus (fig. 3c, d respectively). Additionally, no differences were noted in percent relaxation and stiffness between the HC and HC+S groups (fig. 4a, b respectively).

Histology

In the insertion region, no differences were observed in cell shape (fig. 5a), cellularity (fig. 5b), or CSD (fig. 6) between the HC and HC+S groups. In the midsubstance region, the HC+S group had significantly more spindle shaped cells compared to the HC group (fig. 5a). No significant differences were observed in cellularity (fig. 5b) or CSD (fig. 6) between the HC and HC+S groups. Representative H&E stained sections support these findings (fig. 7).

DISCUSSION

This study examined the biomechanical and histological effects of statin treatment on the rat supraspinatus tendon in a diet-induced hypercholesterolemia model. Results suggest that treating HC rats with daily simvastatin for three months recovers the changes due to HC alone to baseline control properties. Specifically, in the insertion region, elastic modulus was significantly decreased (42%) in the HC+S group compared to the HC group (fig. 3b). Typically, a decrease in elastic modulus might be interpreted as a deleterious outcome, but when considering the mean modulus values in the CTL and HC+S groups (118 ± 47 MPa and 99 ± 30 MPa, respectively), it is apparent that the simvastatin treatment in fact returned elastic modulus values close to baseline control values, which were elevated in the HC group compared to the CTL group (172 ± 35 MPa) (fig. 3b). This finding is consistent with previous findings, with an increase in elastic modulus in the supraspinatus tendon of rats after being fed the HC diet for six months⁶.

Insertion region cross-sectional area was significantly increased (18%) in the HC+S group compared to the HC group (fig. 1a). Increased cross-sectional area is often associated with tendon fibrosis in response to injury and results in inferior mechanical parameters. This finding suggests an alteration in tendon structure, but did not negatively affect clinically relevant mechanical parameters, so another process must be taking place. One potential explanation is that HC caused metabolic changes to the tendon, so the increase in area after simvastatin intervention might be a result of a metabolic repair process such as matrix turnover, though this was not investigated in the current study. Future studies might elucidate this by examining MMP and TIMP activity during simvastatin treatment.

Histological analysis showed the HC+S group had significantly more spindle shaped cells in the midsubstance region compared to the HC group. Again, the same pattern is evident in which the simvastatin intervention helps to bring the tissue closer to baseline values. In tendon tissue, rounded cells are associated with tendinopathy and a more cartilaginous phenotype, which are risk factors for tendon rupture²⁸. Rounded cells are often more metabolically active^{28; 29}, which could be a result of high-cholesterol in the tendon, which was mitigated by simvastatin treatment. A similar pattern (although not statistically significant) is noted in the number of cells in the insertion region where the median grade in the HC group trended toward more cells, but the HC+S group had a trend toward less cells compared to the HC group, with the median grade in the HC+S group being the same as the

median grade in the CTL group. Histological properties display similar patterns as biomechanical parameters, with the simvastatin intervention helping to restore tendon properties closer to baseline control properties.

Statistical differences were not detected in collagen fiber organization at either the insertion or midsubstance region. Typically, as the tendon approaches the transition zone, collagen fiber organization decreases and the variability in data increases. This might explain why it is often difficult to detect differences in the insertion region. A previous study in our lab examining healing tissue in rats fed an HC diet also found no differences in collagen fiber organization in HC vs CTL tendons⁷, consistent with our findings.

Animal weights in the CTL group were significantly larger than the HC and HC+S groups (fig. 2), although the percentage of weight gained each week was similar in each group (fig. 2). This is consistent with previous findings⁷, suggesting that the HC diet might be slightly less palatable than the standard rat chow. Additionally, differences in weight might be explained by the neophobic nature of rats, although it would be expected that the rat weights would merge over time once the animals acclimated to the HC diet. Importantly, no differences were observed in weight between the HC and HC+S groups, so comparisons between structural parameters are considered appropriate.

Serum lipid panels at 6 months showed significant elevations in TC, HDL, and TC/HDL, but the TG concentration was significantly decreased in the pooled HC groups compared to the CTL group. This demonstrates that the HC diet does in fact induce a hypercholesterolemic state. The decrease in TG concentration is consistent with previous findings, although not significant^{6, 7}. As observed in the biomechanical and histological data, the HC+S group had serum lipid levels closer to the CTL group. Although HC+S values do not approach baseline as drastically as the tendon outcome measurements, this small change in serum lipid levels did result in statistically significant alterations at the tendon level. Future studies might warrant a dose larger than 20mg/kg or longer duration of treatment to further lower serum lipid levels. Of note, additionally, the animals in the HC+S group were still fed the HC diet after simvastatin intervention, which might not exactly mimic the clinical scenario, as patients are typically advised to make dietary changes upon diagnoses of hypercholesterolemia. Perhaps feeding the HC+S group normal chow or a smaller concentration of cholesterol at the induction of simvastatin treatment would result in an additive effect. For this controlled laboratory study, we chose to keep the diets constant to compare the results of the drug treatment alone.

It is important to note that the simvastatin intervention did not negatively affect biomechanical or histological outcomes in a high-cholesterol environment, which, even though commonly prescribed, had not yet been studied. This suggests that simvastatin does not add to the increased risk of tendon rupture in the high-cholesterol population and in fact might help to decrease the prevalence of tendon rupture if high-cholesterol is diagnosed and treated prior to the development of tendon-related pathologies.

This study has several potential limitations. First, an animal model was used to address our research question. Although animal models are a useful tool for understanding physiological

processes, they do not directly mimic the clinical scenario. Secondly, the HC model used in this study was diet-induced. Clinically, genetic factors can also play a role in high-cholesterol, which suggests an alternate pathway in the development of high-cholesterol aside from eating cholesterol rich food. Additionally, only one time point was examined. Simvastatin treatment for more extended periods might strengthen the results found in the current study. Also, only one type of statin was used. There are several varieties of statins available and prescribed, and their effects on tendon properties might vary. An additional control group examining control diet with statin treatment could have been tested, but is currently beyond the scope of this study. Lastly, only the supraspinatus tendon was evaluated in this study. It would be advantageous to examine other commonly injured tendons such as the Achilles and patellar tendons, as they experience different loading environments and might respond differently to simvastatin treatment.

In conclusion, our results suggest that three months of simvastatin treatment in a diet-induced hypercholesterolemia model improves tendon mechanical and histological properties. Additionally, this data suggests that simvastatin use does not negatively affect tendon mechanical properties and might help to reduce the risk of tendon rupture.

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Figure 1.

Overall study design of three groups. CTL= control group, HC= high-cholesterol diet, HC +S= high-cholesterol diet with statin treatment. All animals were sacrificed after 9 months, where left supraspinatus tendons underwent mechanical testing and right supraspinatus tendons were examined histologically.

Figure 2.

Weight over time. Rats in the CTL group had significantly higher body weights compared to the HC and HC+S groups at weeks 1–37. Mean % weight gain each week was not significantly different between groups (CTL mean= $10\% \pm 11\%$, HC mean= $7\% \pm 9\%$, HC+S mean= $7\% \pm 10\%$, $p=0.3$). Statin dosing in the HC+S group begins at vertical line. Data as mean $\pm$ SD.

Figure 3.

Tendon mechanical properties. (A) Insertion cross-sectional area was significantly increased in the HC+S group compared to the HC group ($p=0.01$) (CTL=1.76 mm²±0.42, HC=1.74 mm²±0.29, HC+S=2.06 mm²±0.17). (B) Insertion modulus was significantly decreased in the HC+S group compared to the HC group ($p=0.04$) (CTL=117.66 MPa±46.68, HC=172.42 MPa±95.25, HC+S=99.36 MPa±30.34). (C) No differences were observed in midsubstance cross-sectional area (CTL=1.49 mm²±0.37, HC=1.53 mm²±0.39, HC+S=1.51 mm²±0.25). (D) No differences were observed in midsubstance modulus (CTL=245.26 MPa±119.17, HC=304.27 MPa±118.23, HC+S=282.42 MPa±112.85). Data as mean ± SD.

Figure 4.

Tendon mechanical properties. (A) No differences were observed in percent relaxation (CTL=43%±7, HC=44%±7, HC+S=43%±2). (B) No differences were observed in stiffness (CTL=35.13 N/mm±4.62, HC=29.85 N/mm±7.38, HC+S=32.04 N/mm±7.39). Data as mean ± SD.

Figure 5.

Tendon semi-quantitative histology.(A) No differences were observed in cell shape at the insertion region. In the midsubstance, the HC+S group had significantly more spindle shaped cells compared to the HC group ($p=0.01$). (B) No differences were observed in cellularity at the insertion and midsubstance regions. Data as median $\pm$ IQR.

Figure 6.

Tendon collagen fiber organization. No differences were observed in circular standard deviation at the insertion (CTL=13.76 ± 5.8, HC=12.85 ± 3.47, HC+S=15.82 ± 10.83) and midsubstance (CTL=5.48 ± 1.45, HC=8.03 ± 3.94, HC+S=7.51 ± 3.68) regions. Data as mean ± SD.

Figure 7.
Representative histology images. INS= insertion region, MID= midsubstance. CTL= control, HC= high cholesterol diet, HC+S= high cholesterol diet with statin treatment.

Table 1**Serum Lipid Levels**

Serum lipid panel showing serum at month 6 (after induction of HC). HC and HC+S data is pooled at 6 months because they received the same treatment up until 6 months. Pooled HC and HC+S groups has significantly increase TC, HDL, and TC/HDL and significantly decreased TG compared to CTL group. Serum lipid panel at month 9 (after 3 months of simvastatin treatment in the HC+S group) showed HC+S group had significantly decreased HDL and a trend toward decreased TC compared to the HC group. No differences were observed in TG or TC/HDL when comparing HC and HC+S groups.

	TC (mg/dL)	HDL (mg/dL)	TG (mg/dL)	TC/HDL
Month 6				
CTL	90.8±14	50.6±9	152±44	1.8±0.1
HC	269±81	82.1±18	82±16	3.2±0.4
HC+S	252±74	69.5±11	77.4±18	3.3±0.3
P value (CTL vs HC pooled)	<0.001	<0.001	<0.001	<0.001
Month 9				
CTL	106±13	61.4±9	181±26	2.0±0.6
HC	393±113	115±29	144±42	3.4±0.4
HC+S	323±113	91.1±24	130±58	3.5±0.5
P value (HC vs HC+S)	0.1	0.03	0.3	0.3