Simvastatin does not adversely affect Achilles tendon properties in a diet induced hypercholesterolemia rat model

Daniel S. Choi
Snehal S. Shetye
Jennica J. Tucker
Julianne Huegel
Louis J. Soslowsky

McKay Orthopaedic Research Laboratory, University of Pennsylvania, Philadelphia, Pennsylvania, USA

Corresponging author:
Louis J. Soslowsky
McKay Orthopaedic Research Laboratory
University of Pennsylvania
110 Stemmler Hall
3450 Hamilton Walk
Philadelphia, PA, USA 19104
Tel.: +12158988653
E-mail: soslowsk@upenn.edu

Summary

***Introduction*: Hypercholesterolemia affects a third of adults in the United States and is commonly managed with statins. While statins provide proven cardiovascular benefits, their effect on the musculoskeletal system has been understudied. The purpose of this study was to investigate the effects of statins on Achilles tendons of diet induced hypercholesterolemic rats.**
***Methods*: Thirty adult male Sprague-Dawley rats were assigned to three groups: control (CTL, n=10), high cholesterol (HC, n=10), and high cholesterol with statin (HC+S, n=10). CTL was fed normal chow, while HC and HC+S were fed high cholesterol chow. HC+S received simvastatin during the final 3 months, with all animals sacrificed 9 months after the study was initiated, followed by biomechanical testing and histological analysis.**
***Results*: HC Achilles demonstrated increased stiffness as compared to both CTL and HC+S. Histological analysis revealed trends toward increased cellularity in HC as compared to both CTL and HC+S. Both stiffness and cellularity exhibited a potential return to baseline effect with statin treatment, with no differences observed between CTL and HC+S.**
***Conclusions*: While we were unable to determine conclusive therapeutic effects of statins, results support that statins may not have detrimental effects on Achilles tendon health when prescribed to hypercholesterolemic individuals.**
Level of Evidence: Not applicable, this is a basic science study.

KEY WORDS: animal model, biomechanics, orthopaedics, statins.

Introduction

Hypercholesterolemia affects more than 32% of adults in the United States[1]. In addition to increasing the risk for heart disease, stroke, and peripheral artery disease, previous studies have shown hypercholesterolemia to be a risk factor for tendon ruptures in both the supraspinatus and Achilles tendons[2]. Hypercholesterolemia has deleterious effects on tendons due to both the deposition of cholesterol byproducts in the tissue and alterations in the composition of the tendon extracellular matrix[3,4]. Lipid depositions commonly lead to the formation of tendon xanthoma nodules, which disrupt normal tendon structure, reduce biomechanical properties, and increase risk of tendon rupture[3]. Elevated cholesterol levels were identified in 83% of patients with Achilles tendon ruptures[5]. We have previously reported increased stiffness and elastic modulus in the supraspinatus tendon in animals eating a high-cholesterol diet for 6 months[6]. In an otherwise healthy tendon condition, such alterations in tendon properties may lead to chronic tendinopathy and rupture.
Statins are a class of lipid-lowering medications that are commonly administered for the treatment of hypercholesterolemia, and have been found to reduce the risk of cardiovascular disease[7]. However, their effect on the musculoskeletal system, particularly on those subjected to the systemic influence of high cholesterol, are relatively unknown.
Results of retrospective clinical studies investigating the effects of statin therapy in hypercholesterolemic patients are contradictive. Some suggest a link between statin treatment and the incidence of tendinopathy and tendon ruptures[8-10], possibly due to an inhibition of type I collagen synthesis[11]. Others have found little to no association between statin therapy and tendinopathy[12-14]. There is also some evidence

suggesting that statins may have a protective effect against rotator cuff injuries[15]. Interpretation of these results is further complicated by the presence of confounding variables such as diabetes mellitus and hyperuricaemia[14]. Such conditions have also been associated with adverse effects in tendons and makes isolating the influence of statin therapy challenging[14,16].

The individual effects of either hypercholesterolemia or statins on tendons have been described by a number of studies in animal models. We have previously reported increased stiffness and modulus of the supraspinatus tendon in several animal models as a result of hypercholesterolemia, including mice (ApoE knockout), rats (diet induced), and non-human primates (diet induced) [6]. Statin therapy alone decreased max load, stress, and modulus of the Achilles tendon in multiple independent rat studies[17,18]. However, very few studies have investigated the role of statin therapy in the presence of hypercholesterolemia. Clinically, patients are prescribed statins in response to hypercholesterolemia; therefore, it is important to understand the combined effect of statin and high cholesterol. Previous work from our lab investigated the effects of statin on the rat supraspinatus tendon in a diet induced high cholesterol model. Surprisingly, this study did not demonstrate diminished tendon properties due to statin in such a high cholesterol model; therefore, this study did not replicate the results obtained after statin therapy alone[17]. As the supraspinatus and Achilles tendons differ in a number of ways, including the loading environment and extent of vascularity, it is likely that hypercholesterolemia and statin therapy would affect these tissues differently. Therefore, the purpose of this study was to elucidate the effects of statins on the Achilles tendons of the same diet induced hypercholesterolemic rats. Since previous studies suggest that hypercholesterolemia increases tendon properties such as stiffness, and statin therapy decreases tendon mechanical properties, we hypothesized that statin treatment in rats with hypercholesterolemia would improve the mechanical and histological properties of their Achilles tendons by restoring them towards control values.

Materials and methods

Study Design: Achilles tendons were collected from 30 adult male age-matched Sprague-Dawley rats (400-450 g). Supraspinatus tendons had been previously harvested from these rats in a study approved by the University of Pennsylvania's Institutional Animal Use and Care Committee, and methods regarding animal use were previously published[19]. Briefly, rats were housed in pairs in a conventional facility with 12h light/dark cycles. Over the course of 9-months, 20 rats received a high cholesterol diet to induce hypercholesterolemia, while the remaining 10 rats received standard rat chow. The high cholesterol diet consisted of specially formulated rat chow with 4% cholesterol and 1% sodium cholate (Research Diets, Inc., New Brunswick, NJ, catalog #C12595). This diet induced hypercholesterolemia rat model has previously been used and validated in our lab[6,20]. All rats had free access to food and water and underwent weekly weight measurements. After the initial 6 months, a subset (n=10) of the high cholesterol diet rats were orally administered a daily dose of simvastatin (20 mg/kg, Cadila Pharmaceuticals, Ahmedabad, India) for the final 3 months while maintaining a high cholesterol diet (HC+S). This dosing procedure has been previously employed in our laboratory[19]. The remaining rats did not receive statin, but remained on their high cholesterol diets for the final 3 months (HC, n=10). Rats receiving the standard diet served as controls (CTL, n=10). Blood samples were taken from all rats at 6 and 9 months to measure their levels of total cholesterol (TC), high-density lipoprotein (HDL), triglycerides (TG), and TC to HDL ratios (TC/HDL) in order to assess hypercholesterolemia. All rats were sacrificed at the end of the 9 months and stored at -20° C. This study was conducted in accordance with the ethical standards required by this Journal and outlined by Padulo et al.[21].

Tendon Mechanical Testing: Prior to mechanical testing, the specimens were thawed and their left Achilles tendon-foot complexes were removed *en bloc.* Specimens were then fine dissected, removing all extraneous soft tissue including the plantaris longus tendon and the gastrocnemius/soleus muscles. Verhoeff's stain was used to transversely mark the tendons at the tendon insertion and at the testing gauge length of 12 mm proximal to the insertion. A custom laser device was used to measure tendon cross sectional area[22]. Foot tissue including the calcaneus was then embedded in polymethyl methacrylate bone cement. The free end of the tendon was glued between two pieces of fine 400 grit sandpaper using cyanoacrylate adhesive placed at the proximal stain line, preserving the 12 mm gauge length. Specimens were tested using custom fixtures that held bone and tendon tissue in a perpendicular configuration resembling physiological conditions. Specimens were submerged in 37°C phosphate buffered saline solution and tested using an Instron 5543 (Instron, Norwood, MA) equipped with a 500N load cell. The mechanical testing protocol consisted of the following: preload to 0.1N, preconditioning for 10 cycles (0.1 to 0.5N at 1% strain/s), a 300s hold, stress relaxation at 5% strain magnitude for 600s, and ramp to failure at 0.3% strain/s. Images were captured during the ramp to failure portion of the testing sequence. Custom MATLAB software (Mathworks; Natick, MA) was then used to calculate 2D Lagrangian strain by optical tracking of the stain lines[4]. Calculated parameters included cross-sectional area, percent stress relaxation, maximum stress, maximum load, stiffness, and modulus of elasticity.

Histological analysis: Histological analysis was performed on all animals (n=10/group) to assess cell shape and cellularity, proteoglycan content, and presence of calcification or fibrocartilage in the Achilles tendon. At the time of sacrifice, right Achilles musculotendinous units were immediately dissected, fixed in formalin, and processed using standard paraffin procedures. 7µm sagittal sections were prepared and stained with either hematoxylin and eosin to assess cellularity and cell shape at the tendon midsubstance (the region between the bony insertion and the musculotendinous junction) or Safranin O and Fast Green to assess proteoglycan content and localization. Slides were imaged on an AxioZoom slide scanner (Zeiss, Pleasanton, CA), and 200x magnification images were used to assess cellularity and cell shape. Differences in these parameters were determined semi-quantitatively by three blinded graders. The cellularity scale ranged from 1 to 3 with 1 representing the lowest cell density and 3 representing the highest cell density. The cell shape scaled ranged from 1 to 3 with 1 representing classic spindle shaped cells and 3 representing round shaped cells. Grading standards were created within the set of all images, and at least 2 images per region were assessed per specimen. Proteoglycan content and regions of calcification or fibrocartilage were assessed qualitatively.

Statistical analysis: Sample sizes were determined using a priori power analyses. Statistical comparisons were made between all groups for both mechanical and histological assays. Weight, serum lipid panel, and mechanical parameter comparisons were made using one-way ANOVA with significance set at $p \leq 0.05$ and trends at $p \leq 0.1$. *Post hoc t*-tests with Bonferroni corrections were also performed. A non-parametric Kruskal-Wallis ANOVA was used for histological comparisons with significance set at $p \leq 0.05$ and trends at $p \leq 0.1$. *Post hoc* Mann-Whitney *U* tests with Bonferroni corrections were also performed.

Results

Weight & Lipid Panel: Rats on the high cholesterol diet weighed significantly less than those on the regular diet starting one week after the change in diet. No differences in weight were found between the HC and HC+S groups at any time point (data previously reported in Tucker et al.[19]). When comparing high cholesterol groups (HC and HC+S, pooled) to controls at six months, serum lipid analysis found significantly elevated levels of TC (261±76 *vs* 91±14 mg/dl) and HDL (77±17 *vs* 51±9 mg/dl), an increased TC/HDL ratio (3±0.3 *vs* 1.8±0.1), and significantly decreased levels of TG (80±17 *vs* 152±44 mg/dl). After three months of statin treatment, the HC+S group exhibited a trend toward decreased TC (323±113 *vs* 393±113 mg/dl), significantly decreased HDL (91±24 *vs* 115±29 mg/dl), and no significant differences in TG (130± 58 *vs* 144± 42 mg/dl) or TC/HDL ratio (3.5±0.5 *vs* 3.4±0.4) when compared to the HC group[19].

Mechanical Testing: No significant differences were found in tendon cross-sectional area (Fig. 1A) or maximum load (Fig. 1B). Tendon stiffness trended toward an increase in the HC group compared to the CTL group. However, stiffness was significantly decreased in the HC+S group when compared to the HC group (Fig. 1C). Stiffness was not different between CTL and HC+S groups. No differences were found in material or viscoelastic properties, including elastic modulus (Fig. 2A), maximum stress (Fig. 2B), and percent relaxation (Fig. 2C). With the exception of one tendon, all specimens failed at the tendon insertion. The remaining tendon failed at the grip and was considered to be a non-physiological failure. As a result, the data from this specimen was omitted from maximum stress and maximum load calculations.

Histological analysis: In the tendon mid-substance, there were no significant changes in cell shape (Fig.

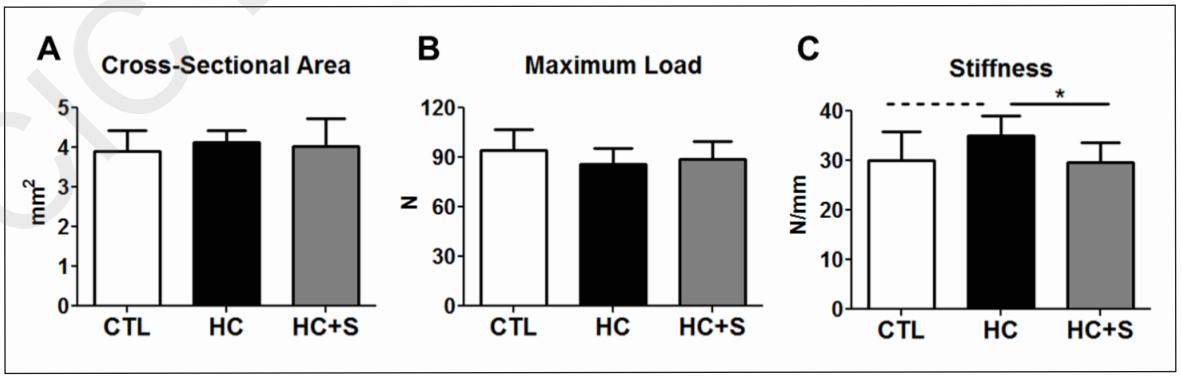

Figure 1. Tendon structural properties at 9 months. (A) No differences in cross-sectional area. (B) No differences in maximum load. Data presented as mean ± standard deviation. Dotted line indicates trends with $p \leq 0.1$, and solid line/asterisk indicates $p<0.05$.(C) Stiffness trended toward increased in the high cholesterol (HC) group compared to controls (CTL) ($p=0.03$) and was decreased in the statin therapy group (HC+S) compared to HC ($p=0.006$).

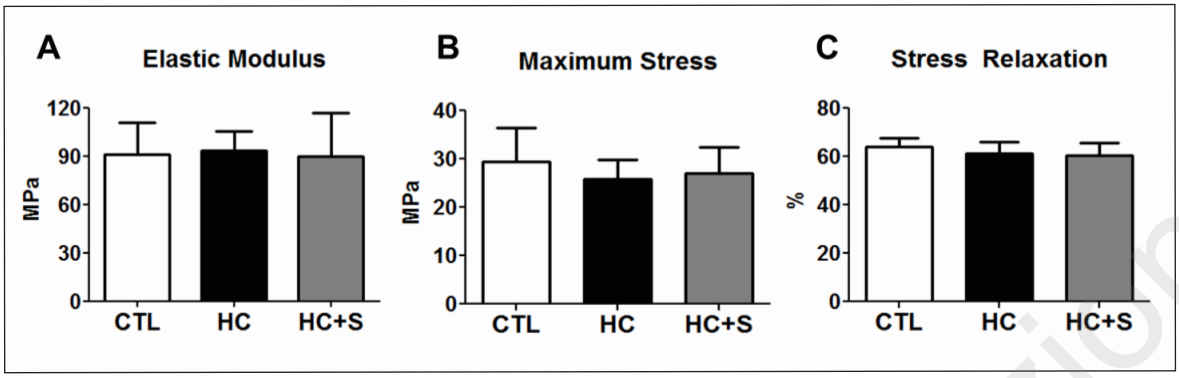

Figure 2. Tendon material and viscoelastic properties at 9 months. No differences in (A) elastic modulus, (B) maximum stress, or (C) stress relaxation across groups. Data presented as mean ± standard deviation. CTL (control); HC (high cholesterol); HC+S (high cholesterol + statins).

3A). There was a trend toward an increase in cell density in the HC group when compared to both the CTL and HC+S groups, but no difference was observed between CTL and HC+S groups. Representative images are shown in Figure 4. Some tendons exhibited small calcification foci (Fig. 5A, B, insets) and/or small areas of fibrocartilage anteriorly (Fig. 5C, D, insets) as noted with both darker eosin staining and increased SafO-positive proteoglycans. However, none of these morphological observations were unique to a particular group, and all were seen in controls. No other morphological changes were identified in any samples.

Discussion

This study investigated the effects of statin treatment in a diet induced hypercholesterolemia model of the rat Achilles tendon. Tendons in the HC group trended toward increased stiffness when compared to the CTL group tendons. This is consistent with previous studies indicating that hypercholesterolemia has detrimental effects on tendon mechanical properties[6]. After 3 months of simvastatin treatment following a high cholesterol diet, the stiffness values of tendons in the HC+S group were significantly decreased as compared to those in the HC group. No significant dif-

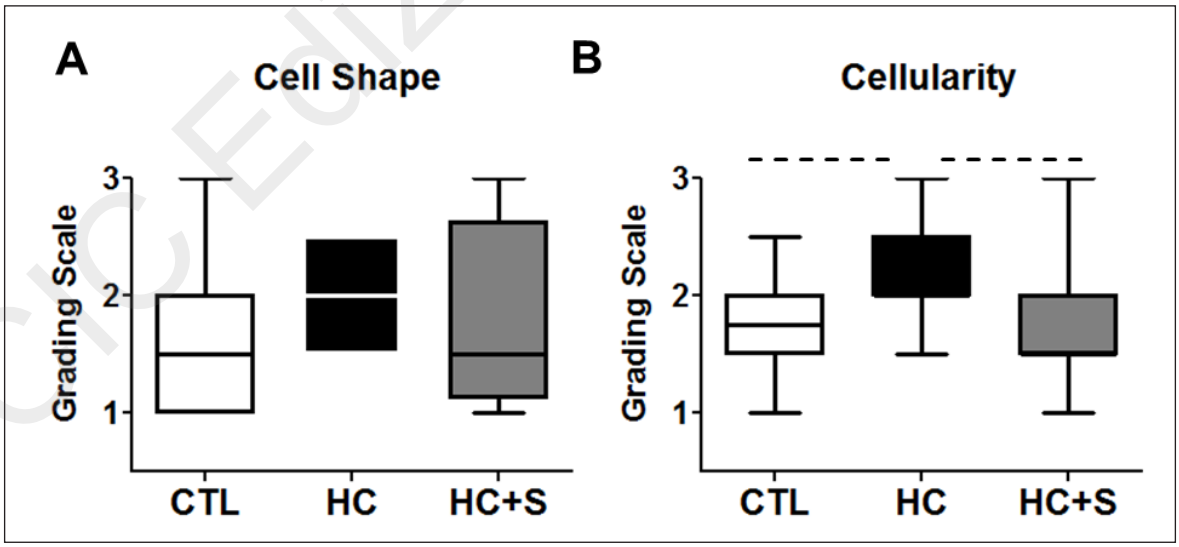

Figure 3. Semi-quantitative histological analysis of the Achilles midsubstance. (A) No differences were observed in cell shape. (B) The high cholesterol (HC) group trended toward increased cellularity compared to both the control (CTL) group (p= 0.03) and the high cholesterol + statins (HC+S) group (p=0.03), but there were no differences between the CTL and HC+S groups. Data presented as median ± interquartile range with maximum and minimum tails. Dotted line indicates trends with p≤0.1.

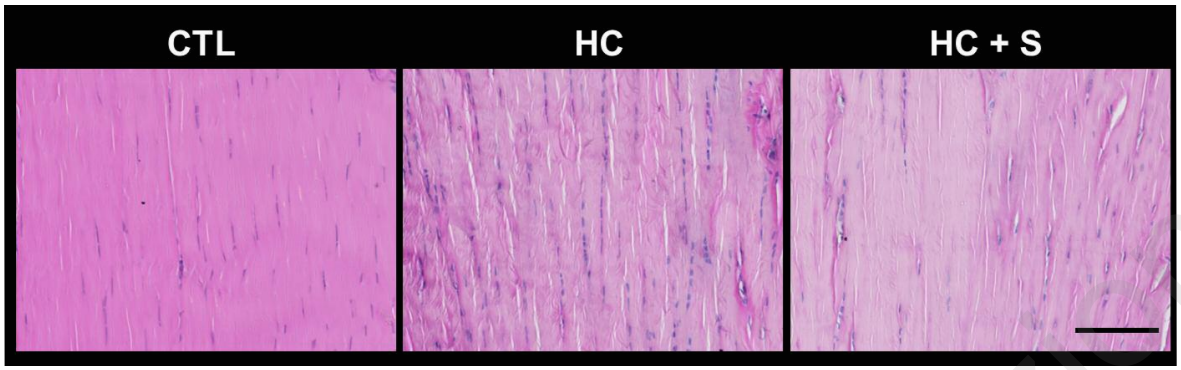

Figure 4. Representative histology images. CTL, control; HC, high cholesterol diet; HC+S, high cholesterol diet with statin treatment. Scale bar: 100 µm.

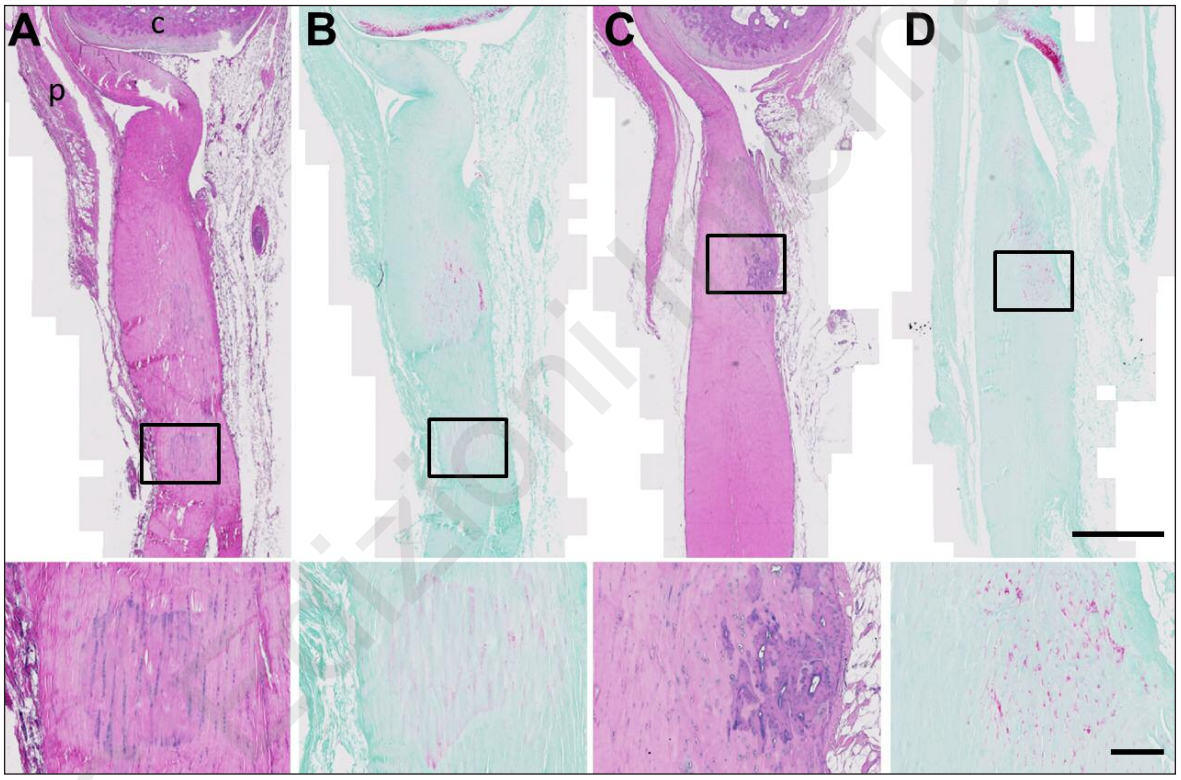

Figure 5. Histological features seen in the Achilles midsubstance. Hematoxylin & eosin staining of representative images show the presence of calcification foci (A) and fibrocartilage (C) in the tendon midsubstance across groups. Serial sections stained with SafraninO-FastGreen indicate increased proteoglycan content in both calcification foci (B) and fibrocartilage (D). Higher magnification of boxed regions shown in insets. Scale bar in D, 3 mm; scale bar in D (inset), 250 µm. c, cartilage surface of calcaneus; p, plantaris.

ferences were seen between CTL and HC+S. Therefore, our results suggest that in the presence of hypercholesterolemia, statins may play a therapeutic role by decreasing stiffness values to a level similar to that of healthy CTL group values**.** No other mechanical parameters were significantly altered in either treatment group.

Interestingly, no differences in tendon cross sectional area were found in this study. Differences were expected as prolonged hypercholesterolemia has been known to increase tendon thickness, specifically in the human Achilles tendon[24]. It has been proposed that increased Achilles tendon thickness could even be an alternative method for diagnosing familial hypercholesterolemia[24]. Although such changes were not found in this study, the difference in body weights of the CTL group *versus* those of the high cholesterol group may be at least partially attributable; conse-

quently this variable cannot be fully evaluated in this study. However, it is possible that rats are not symptomatic in the same manner that humans are. Additionally, tendon failure load and modulus have been shown to decrease after statin treatment in the absence of induced hypercholesterolemia[18]. There were some important differences in study design, particularly in the duration of statin treatment and the lack of a hypercholesterolemic state prior to treatment. Therefore, the lack of differences from our study suggests that in the presence of high cholesterol, statins affect tendon mechanics differently and are less likely to contribute to an overall decrease in tendon health.

Histological analysis also uncovered changes between groups. Cell density, or cellularity, trended toward an increase in the HC group compared with the CTL group. Tendon hypercellularity and active proliferation are classical characteristics of an altered or degenerative tendon state[25]. This finding may correlate with the increased tendon stiffness in the HC group. Additionally, cell density was also decreased in the HC+S group compared with the HC group, indicating a potential return to control level cellularity after statin treatment. Likewise, this trend also corresponds with a decrease in stiffness in the HC+S group. A similar trend was seen in the supraspinatus insertion of these animals[19], where cellularity increased with the high cholesterol diet. However, the addition of statin therapy appears to ameliorate this difference. Therefore, histological properties in this model should be interpreted as beneficial as a result of simvastatin therapy.

Some morphological changes were also noted in the tendon midsubstance, including small calcification foci and areas of fibrocartilage. Fibrocartilage was always localized to the anterior portion of the tendon, and is considered a normal phenomenon due to the adjacent prominent anterior tuberosity of the calcaneus and the associated compression in this region[26]. These features were noted in all groups, including controls, and were not more prevalent in any specific group. *In vitro* studies have demonstrated changes in matrix production due to statin treatment[27], but our findings did not support a notable change in proteoglycan content with any treatment condition.

In this study, we found consistent and significant differences in weights of the animals in the CTL group versus those in the HC and HC+S groups. Rats on high cholesterol diets weighed significantly less than those on the regular diet. This suggests that the HC diet may be less palatable than standard rat chow. However, the percentage of weight gained each week remained similar between groups, consistent with previous findings[6]. Rats are neophobic by nature, which may explain an initial period of slow growth after initiating the diet, followed by steady weight gain after acclimation to the new diet.

Although the animals experienced the same experimental conditions, several notable differences exist between the previously reported supraspinatus tendon data and the Achilles tendon data from this study. The supraspinatus tendons of the HC+S group were found to have significantly greater cross sectional area as compared to the HC group in the insertion region[19]. Cross sectional area differences were not seen in the Achilles tendon. Further, tendon modulus was decreased in the HC+S group of the supraspinatus, while no changes were observed between groups in the Achilles tendon. Lastly, the differences in stiffness that were detected in the Achilles tendon were not seen in the supraspinatus tendon. This suggests that the combination of hypercholesterolemia and statins affect the mechanical properties of different tendons in different ways. An unrelated study examining several different tendon types from a genetic hypercholesterolemia rabbit model found increased vascularity and mechanical stress to be essential factors in lipid related pathology[28]. As the Achilles tendon differs in both extent of vascularity and loading regimes compared with the supraspinatus tendon, it is possible that these factors influence how these tendon tissues respond to the experimental conditions. This may help to explain our differential findings.

There were several limitations to this study. The most apparent was the use of a diet induced model of hypercholesterolemia, which does not take genetic factors into account. However, dietary hypercholesterolemia is still relevant as most patients present with mixed hypercholesterolemia clinically[29]. Additionally, while the model does not take into consideration dietary changes that patients may make in reaction to a hyperlipidemia diagnosis, studies have shown that the majority of patients are unaware of their condition until after tendon rupture, indicating significant damage incurred[5]. Further, hypercholesterolemia is known to cause coronary heart disease and other systemic changes over a lifetime of exposure. Although, a high cholesterol state was confirmed in the animals by the 6-month blood lipid panels, the total of 9 months of a high cholesterol diet may be inadequate to create significant musculoskeletal changes. Lastly, the duration, dosage, and delivery of statin therapy could be modified. As seen in the 9-month blood lipid panel, the statin treatment did not lead to a pronounced cholesterol reduction to the extent that was expected. However, the diet and statin treatment regimen was appropriate for the model as previously described[17-19].

Additional studies examining tendon properties at further time points or with different dosages of statins may help to further elucidate the effects of statin on tendons in a hypercholesterolemic environment. Furthermore, the incidence of hypercholesterolemia increases with age. Only 11.7% of individuals ages 20-39 are afflicted with high LDL-C as opposed to 58.2% of individuals ages 65 or greater[30]. Taking this into consideration, a high cholesterol model in an aged rat model may aid in magnifying the interrelated influences of hypercholesterolemia and statin therapy.

This study did not detect differences in mechanical and histological properties in Achilles tendons of hy-

percholesterolemic rats treated with statin compared to controls. However, this result could be interpreted as in support of our hypothesis. Perhaps most importantly, the drug was administered at pharmacological doses and no evidence of adverse effects in the Achilles tendon due to such treatment was found. Therefore, results support the possibility that simvastatin treatment may not majorly compromise Achilles tendon health in hypercholesterolemic individuals.

Conflicts of interest

The Author (LJS) has a patent related to hypercholesterolemia and tendinous injuries (US9427437). No other conflicts of interest exist that might be construed as affecting the conduct or reporting of the work presented.

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