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Diabetes Alters Mechanical Properties and Collagen Fiber Re-Alignment in Multiple Mouse Tendons

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Abstract—Tendons function to transfer load from muscle to bone through their complex composition and hierarchical structure, consisting mainly of type I collagen. Recent evidence suggests that type II diabetes may cause alterations in collagen structure, such as irregular fibril morphology and density, which could play a role in the mechanical function of tendons. Using the db/db mouse model of type II diabetes, the diabetic skin was found to have impaired biomechanical properties when compared to the non-diabetic group. The purpose of this study was to assess the effect of diabetes on biomechanics, collagen fiber re-alignment, and biochemistry in three functionally different tendons (Achilles, supraspinatus, patellar) using the db/db mouse model. Results showed that cross-sectional area and stiffness, but not modulus, were significantly reduced in all three tendons. However, the tendon response to load (transition strain, collagen fiber re-alignment) occurred earlier in the mechanical test, contrary to expectations. In addition, the patellar tendon had an altered response to diabetes when compared to the other two tendons, with no changes in fiber re-alignment and decreased collagen content at the midsubstance of the tendon. Overall, type II diabetes alters tendon mechanical properties and the dynamic response to load.

Keywords—Tendon mechanics, Realignment, Type II diabetes, Supraspinatus, Achilles, Patellar, db/db mouse.

INTRODUCTION

Tendons function to transfer load, maintain alignment and permit motion in joints. To perform in this manner, tendons have complex mechanical behavior that exhibits viscoelasticity, nonlinearity and anisotropy.[8,14,31,34,38] This behavior is modulated by the structure and composition of the tissue, which can vary widely between (Achilles vs. patellar) and within tendons (insertion vs. midsubstance).[8,12,13,16] Generally, tendon is made primarily of a hierarchical collagen structure, with collagen fibrils that bundle to form fibers which bundle to form tendon.[36] Collagen fibers, and the surrounding glycosaminoglycans (GAGs), are thought to be the primary load-bearing structures in tendon. Alterations in the collagen structure and composition could lead to changes in functional capacity and ultimately, tendon rupture. Recent studies have shown that many structural changes affect the way tendon responds to load, not only in quasi-static mechanics, but also in the dynamic response to load and in particular re-alignment and uncrimping of the collagen fibers.[7,13,25,26]

Alterations in collagen have been shown to alter the normal function of many organ systems (e.g., cardiovascular disease, blindness, kidney disease).[5,6,9] Recent evidence has shown that Type II diabetes may cause alterations in collagen structure and subsequently mechanical function.[18–20] *In vitro*, collagen organization is altered with the presence of glycation, resulting in increased mechanical properties and irregular fibril morphology and density.[20] The Maillard reaction, which occurs commonly with age but also at an accelerated pace in patients with type II diabetes,[3,15,27] alters collagen structure, resulting in closer packing of collagen molecules and altered fibril morphology.[19,28] We have recently reported that the skin from the db/db mouse, a model of type II Diabetes, and the skin from human diabetics exhibit decreased maximum stress and modulus compared to non-diabetic skin.[4] However, the mechanical function of the tendons in this diabetic mouse model has not yet been studied. While studies have revealed the presence of advanced glycation end-products in tendons of diabetic animal models,[28,29,33] it is also still unclear if there are changes in

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other extracellular matrix proteins that could cause changes in mechanical properties, such as proteoglycans or glycosaminoglycans (GAGs). In addition, collagen fiber re-alignment in response to load, which is indicative of small structural alterations in the tissue, has also not been studied in diabetic tendons.

Therefore, the purpose of this study was to investigate tendon's response to load in the diabetic db/db mouse tendons, specifically by assessing biomechanics, re-alignment, and biochemistry. We studied three different tendons (Achilles, patellar, and supraspinatus) to determine if the effects of diabetes were different across tendons that vary in both structure and mechanical function. We hypothesized that tendon mechanical properties would be reduced in the db/db tendons, re-alignment would be delayed during loading, and that decreases in collagen and glycosaminoglycan content would be present in all three tendons.

MATERIALS AND METHODS

Sample Preparation

Supraspinatus, Achilles, and patellar tendons from 18 sixty-day old mice (8 db/db diabetic mice and 10 db/+ non-diabetic heterozygous control mice) were used in this study (IACUC approved). Tendons from the left limbs were used for mechanical testing and re-alignment analysis, while tendons from the right limbs were used for biochemical assays. For mechanical evaluation, excess soft tissue was removed and stain lines were placed on the tendons to denote the insertion site and midsubstance for optical strain tracking. Cross-sectional area (CSA) was measured using a laser device and the tendons were secured in custom fixtures. For the supraspinatus tendon, the humerus was affixed in a polymethylmethacrylate (PMMA) pot and the tendon was secured in sandpaper *via* cyanoacrylate adhesive. For the Achilles tendon, the calcaneus was held in a custom grip while the tendon was secured *via* sandpaper in the same fashion as the supraspinatus. For the patellar tendon, the tibia was potted in PMMA at a 45° angle while the patella was secured in a custom grip. An angle of 45° was necessary to permit polarized light (collagen alignment) to pass through the insertion site of the tendon without being obscured by the tibial plateau. The gauge lengths were 2.5, 3, and 5 mm for the supraspinatus, patellar, and Achilles tendons, respectively.

Mechanical Testing

Samples were then loaded in a testing system integrated with a polarized light setup, consisting of a backlight, 90°-offset rotating polarizer sheets on either side of the test sample, and a digital camera.[7,24] Tendons were tested in tension along the long axis of the tendon using the following protocol: preload to 0.02 N, 10 cycles of preconditioning between 0.02 N and 0.04 N, return to zero displacement, a stress relaxation test at 5% strain/s, followed by a 60 s hold and ramp to failure at 0.1% strain/s. Images were obtained every 5 s for strain analysis. Additionally, sets of 13 images were acquired every 20 s as the polarizers rotated through a 125° range for measurement of fiber alignment during loading. Local strain was measured optically and stress was calculated as force divided by initial area. Fiber alignment was calculated from the image sets as described previously.[21,22,24,26] Circular variance (VAR), a measure of the distribution of collagen fiber alignment, was calculated for fiber distributions before preconditioning, after preconditioning, at transition strain (intersection of the toe- and linear-regions, determined using a structural fiber recruitment model at 50% fiber recruitment[30]), and at linear-region strain (at 75% fiber recruitment). Fiber re-alignment during preconditioning was evaluated by comparing VAR values before and after preconditioning. Similar methods were used to determine fiber re-alignment in the toe- and linear-regions of the stress–strain curve.

Biochemistry

Tendons designated for biochemistry were dissected for location-dependent analysis. All tendons were removed with muscle intact from the insertion site. The muscle was then removed with a scalpel blade to leave only the tendon portion. A consistent piece of the insertion and the midsubstance was taken from each tendon by splitting the tissue into three even regions and removing the first (insertion site) and third (midsubstance) region. The supraspinatus and patellar tendons allowed for 0.5 mm long pieces, while the Achilles tendon was large enough to obtain a 1-mm piece for each region. Tendons were then digested in a Proteinase K and Ammonium Acetate solution overnight at 37 °C. DNA content and GAG content in the digest were quantified using the PicoGreen and dimethylmethylene blue (DMMB) assays, respectively.[2,32] The remaining digest was hydrolyzed with hydrochloric acid, resuspended in assay buffer and used to quantify collagen using the hydroxyproline (OHP) assay, as previously described.[2,13] GAG and OHP content were normalized to DNA content to account for differences in tissue size between samples.

Statistical Analysis

Statistical comparisons were only made between genotype and location and not across different

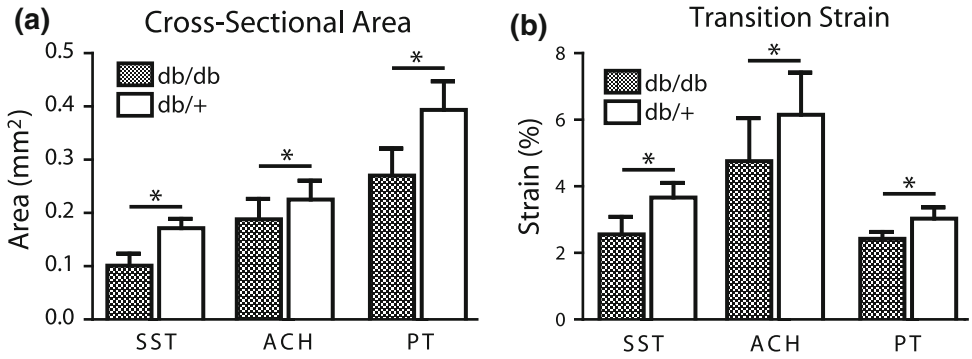

FIGURE 1. (a) Cross-sectional area and (b) transition strain are significantly reduced in the diabetic supraspinatus, Achilles, and patellar tendons.

tendons. Comparisons were made between groups using two-way ANOVAs followed by *post hoc t* tests and Bonferroni corrections for multiple comparisons when location-dependent effects were measured. Student *t* tests alone were used when location-dependent effects were not measured. Non-parametric Wilcoxon signed-rank tests were used for re-alignment analysis, since the data was non-normally distributed. Alignment data is presented as individual samples that represent population results and mechanics and biochemistry data is presented as mean ± standard deviation (*Sig = $p < 0.025$, #Trend = $p < 0.05$).

RESULTS

Supraspinatus Tendon

In the supraspinatus, cross-sectional area was smaller and transition strain occurred earlier in the stress–strain curve in the diabetic group compared to the non-diabetics (Fig. 1). Stiffness was also significantly decreased at the insertion site in the diabetic group (Fig. 2a), but there were no differences in stiffness at the midsubstance or modulus at either location (Fig. 2b). There were no differences between the diabetic and non-diabetic groups for GAG or collagen content, but the non-diabetic group had more GAGs at the insertion site than at the midsubstance, a finding not present in the diabetic group (Fig. 3). Finally, collagen fiber re-alignment occurred during the linear region of the mechanical test for the insertion site and midsubstance of the non-diabetic group (Fig. 4). The diabetic group had re-alignment during the linear region in the midsubstance (same as non-diabetic midsubstance) and during preconditioning and the linear region of the insertion site (different from non-diabetic insertion site).

Achilles Tendon

In the Achilles tendon, cross-sectional area was smaller and transition strain occurred earlier in the stress–strain curve in the diabetic group (Fig. 1). Stiffness and modulus were also both significantly decreased in the diabetic group at the insertion site, but not at the midsubstance (Fig. 2). Compositionally, there were no differences between the diabetic and non-diabetic groups for GAG or collagen content and the midsubstance had more GAG and collagen than the insertion site for both groups (Fig. 3). In the non-diabetic tendons, re-alignment occurred during preconditioning and during the linear region of the mechanical test at the midsubstance and during preconditioning and in the toe region at the midsubstance (Fig. 5). In the diabetic tendons, re-alignment occurred during preconditioning and during the linear region at the midsubstance (same as non-diabetic midsubstance) but only during preconditioning at the insertion site (different from non-diabetic insertion site).

Patellar Tendon

The diabetic patellar tendons were also significantly smaller than the non-diabetics and the transition strain occurred significantly earlier in the stress–strain curve (Fig. 1). Similar to the supraspinatus, the stiffness at the insertion site was decreased in the diabetic group but there were no changes in midsubstance stiffness or modulus at either location (Fig. 2). There were also no differences in group or location in GAG content but, the midsubstance of the non-diabetic group had more collagen than the insertion and it was significantly greater than the diabetic midsubstance as well (Fig. 3). Finally, re-alignment occurred during the linear region of the mechanical test only for both groups at both locations (Fig. 6).

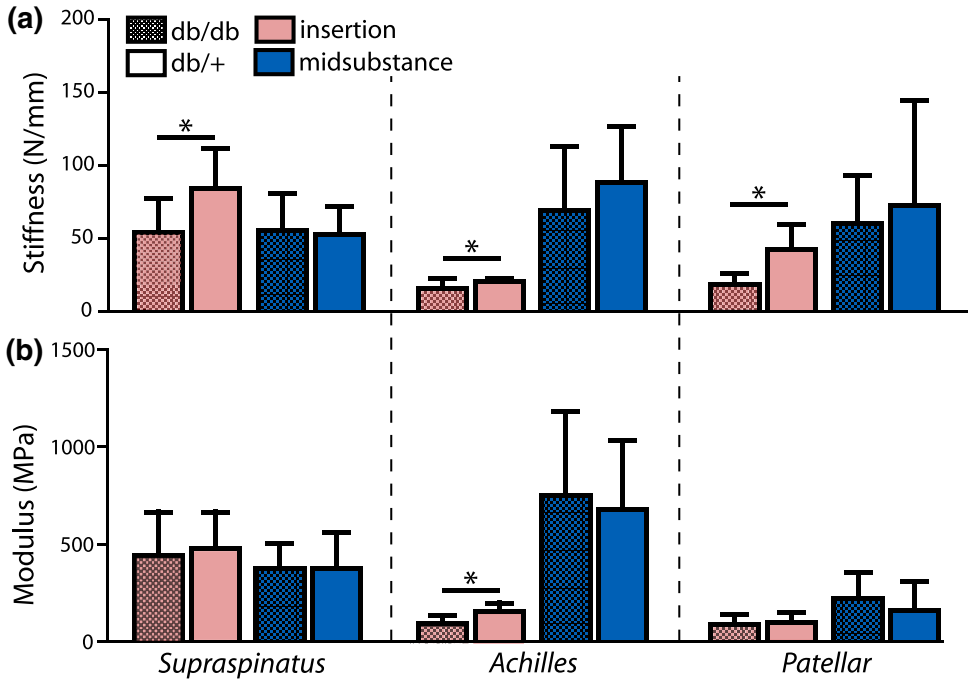

FIGURE 2. (a) Stiffness at the insertion site is significantly reduced in the diabetic tendons for all three tendons. (b) Modulus is also significantly reduced with diabetes in the Achilles tendon. Midsubstance results show no differences between the groups in stiffness or modulus in any tendon.

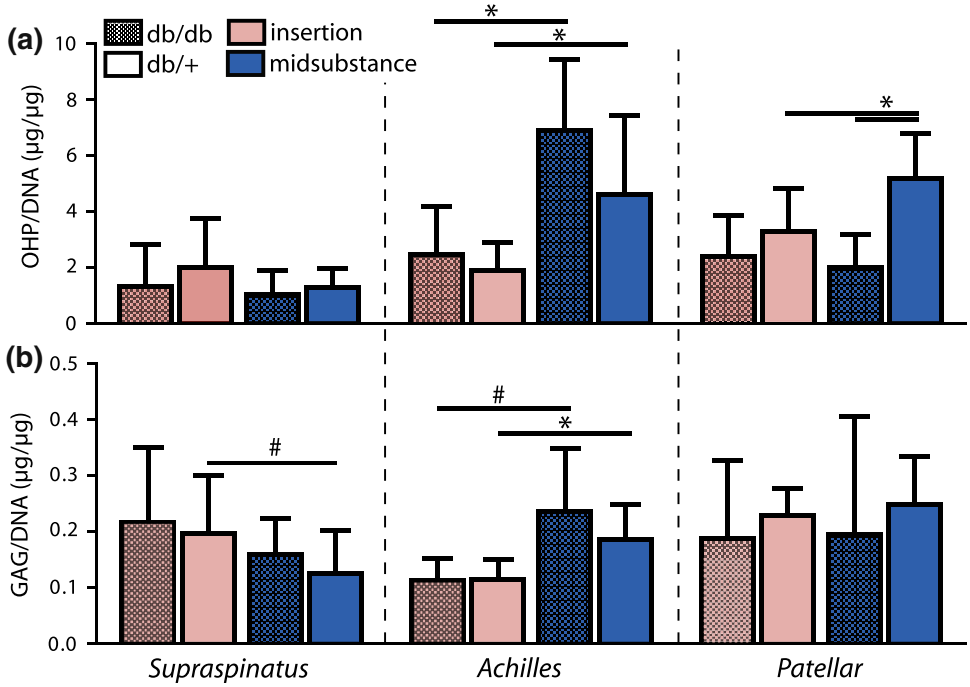

FIGURE 3. (a) The Achilles tendons had significantly more collagen at the midsubstance than the insertion site but there was no difference due to diabetes. The patellar tendons had increased collagen at the midsubstance in the non-diabetic group, which was not present in the diabetic group. In addition, the patellar tendon midsubstance in the diabetes group was significantly lower than the non-diabetic group. (b) GAG content was not different in any group between the non-diabetic and diabetic groups. However, the supraspinatus non-diabetic tendons had more GAGs at the insertion than the midsubstance and the Achilles tendons in both groups had more GAGs at the midsubstance than the insertion site.

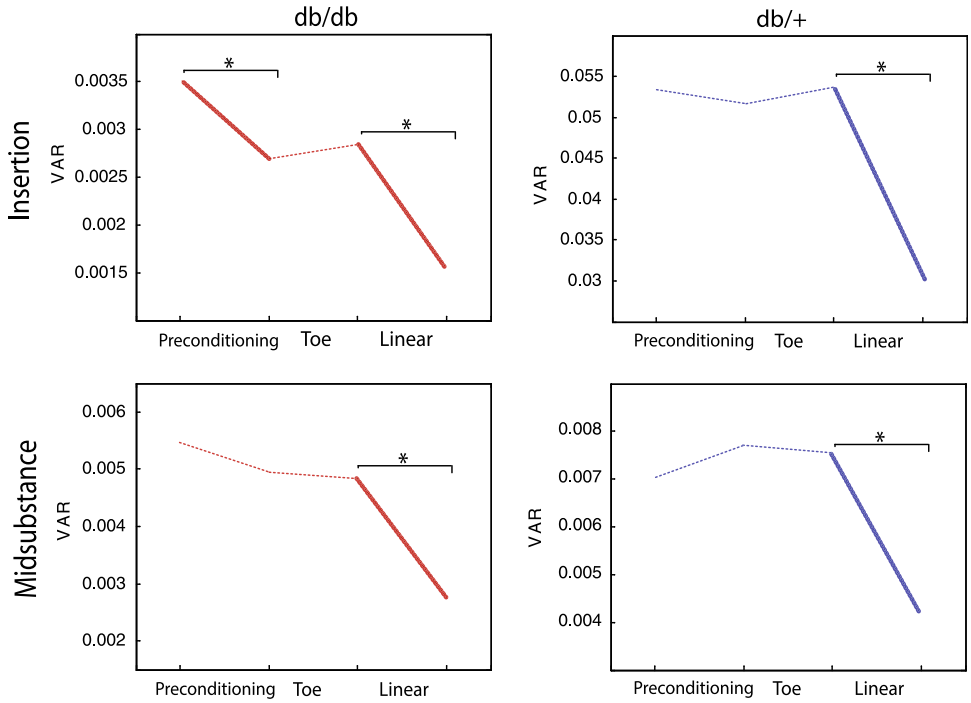

FIGURE 4. At the insertion site, diabetic supraspinatus tendons re-aligned during preconditioning and in the linear region, while only in the linear region in the non-diabetic group. At the midsubstance, there was no difference between the diabetic and non-diabetic groups with re-alignment occurring in the linear region in both groups. Results here show are representative samples showing the population statistics, where the bold lines represent a significant change in circular variance during the region shown on the *y* axis.

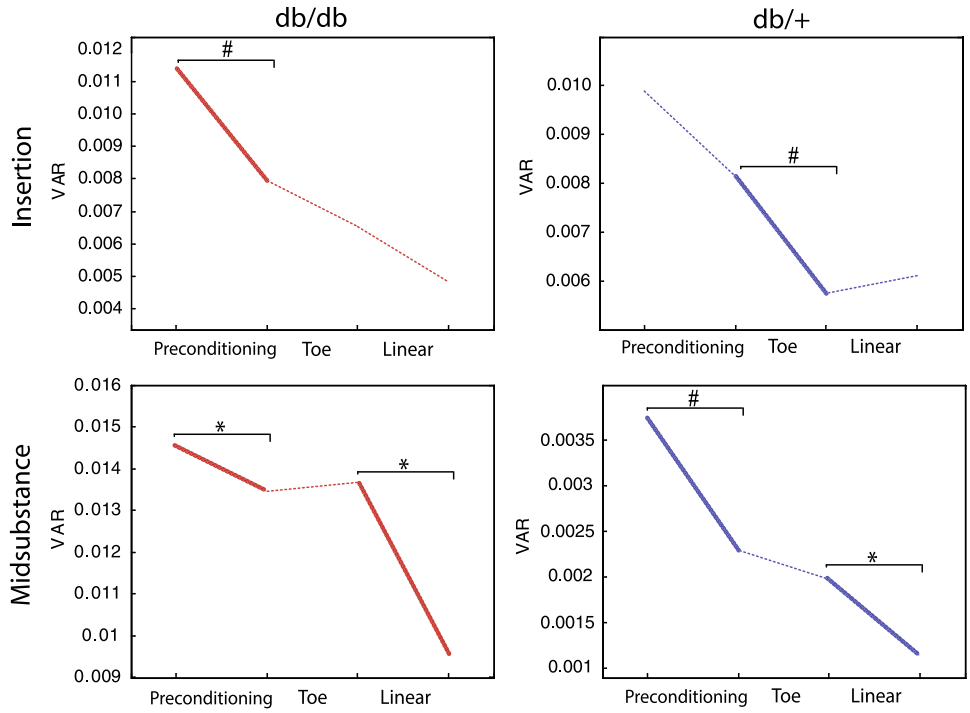

FIGURE 5. At the insertion site, diabetic Achilles tendons re-aligned during preconditioning while the non-diabetic tendons re-aligned during the toe region of the test. At the midsubstance, both groups re-aligned during the preconditioning and linear region of the mechanical test. Results here show are representative samples showing the population statistics, where the bold lines represent a significant change in circular variance during the region shown on the *y* axis.

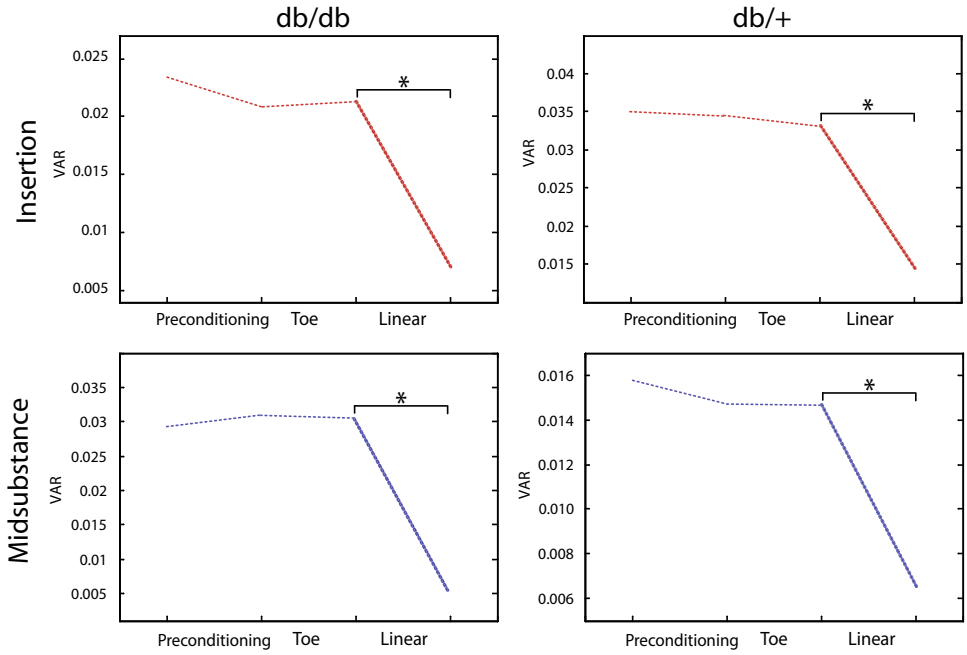

FIGURE 6. In the patellar tendons, there were no significant differences in re-alignment between the non-diabetic and diabetic tendons at either location. Results here show are representative samples showing the population statistics, where the bold lines represent a significant change in circular variance during the region shown on the *y* axis.

		Supraspinatus	Achilles	Patellar
Cross-Sectional Area		↓	↓	↓
Transition Strain		↓	↓	↓
Stiffness	Insertion	↓	↓	↓
	Midsubstance	—	—	—
Modulus	Insertion	—	↓	—
	Midsubstance	—	—	—
Re-Alignment	Insertion	Earlier	Earlier	—
	Midsubstance	—	—	—
Collagen Content	Insertion	—	—	—
	Midsubstance	—	—	↓
GAG Content	Insertion	—	—	—
	Midsubstance	—	—	—

FIGURE 7. Summary of findings for significant changes with diabetes are shown here where a down arrow indicates that the diabetes group was significantly less than the control group in that parameter and a change in re-alignment is noted by 'Earlier', meaning that the diabetes group re-aligned earlier in the mechanical test.

DISCUSSION

This study investigated the role of type II diabetes in tendon mechanical function and composition (Fig. 7). Diabetic tendons were smaller and less stiff than control tendons in the supraspinatus, Achilles, and patellar tendon. These results were consistent with our previous data in the diabetic db/db mouse skin, where the diabetic skin had impaired mechanical properties, specifically reduced maximum stress and modulus.[4] In addition, other studies with induced experimental type I and type II diabetes in the rat have shown reduced mechanical properties when compared to controls.[11,18] However, diabetic tendons had a lower modulus than control tendons only at the Achilles tendon insertion site. While the modulus results suggest an effect of diabetes in only one of the tendons studied, stiffness

measurements show changes in the structural response to load in all three, suggesting that the response to diabetes is more complicated than simple biomechanical measurements (Figs. 2, 7). Interestingly, changes in these parameters were only found at the insertion site of the tendons. This could be due to the insertion site being the initial source of load transfer from bone to tendon and a weaker tissue mechanically, making it more susceptible to mechanical alterations. Moreover, the insertion site is a compositionally and structurally different tissue from the tendon midsubstance and alterations that may exist due to diabetes might play a different role at each location.

Based on the reduction in stiffness, we also expected to find a diminished response to load in the diabetic tendons. However, the results in this study were contrary to this hypothesis. This study found a decreased transition strain in all three diabetic tendons, suggesting an earlier transition to the linear region of the stress–strain curve and thus an earlier response to load. This was also confirmed in our measure of collagen fiber re-alignment in the supraspinatus and Achilles, but not the patellar tendon, which showed an earlier response to load at the insertion site of both tendons. An alteration in the structural response to load could be explained by changes in load-bearing compositional elements such as collagen and GAGs. However, this study found no significant differences in GAG or collagen content between diabetic and non-diabetic Achilles and supraspinatus tendons, indicating that another compositional element must be playing a role. An increase in collagen cross-links, such as the non-enzymatic collagen cross-link pentosidine, has been quantified in previous studies of aged tendon and aged and diabetic skin.[10,15] Several studies have showed increased mechanical response to load with increased pyridinoline, which is an enzymatic crosslink naturally found in abundance in tendon.[10,17,23,35] However, the mechanism by which collagen cross-links affect the tendon mechanical response, particularly in collagen fiber re-alignment, as well as relative role of non-enzymatic and enzymatic crosslinks, is still currently unknown.

Interestingly, the patellar tendon did not show the same changes as the supraspinatus and Achilles in fiber re-alignment when comparing the diabetic and non-diabetic groups. There was no effect of diabetes on the collagen fiber re-alignment at the insertion or midsubstance of the patellar tendon, suggesting that the insertion of the patellar tendon may be different from the insertion of the supraspinatus and Achilles tendons in its mechanical response to load, and that diabetes may have a smaller effect on the patellar tendon than the other two tendons. Since the supraspinatus, Achilles, and patellar tendons all perform different functions and likely have variation in both structure and composition at some level in order to perform those functions, it's likely that natural differences in tendon composition and structure are responsible for these changes. Moreover, the patellar tendon spans from the tibia to the patella, having an interface with two bones instead of bone and muscle like the supraspinatus and Achilles, thus complicating the comparison of results.

The biochemical response to diabetes was also different in the patellar tendon than in the Achilles or supraspinatus tendons. The patellar tendon midsubstance showed an effect of diabetes on collagen content which was not seen in the Achilles or supraspinatus, where the collagen was significantly reduced at the midsubstance of the diabetic tendons. In addition, there was no regional dependence of GAG content in the patellar tendon as was measured in the diabetic (Achilles, supraspinatus) and non-diabetic (Achilles only) groups for the other two tendons. Results in GAG content may indicate that the patellar tendon insertion and midsubstance may be more similar in composition or require more similar functional needs than in the other two tendons, although no studies have investigated this to date. Studies that have compared biochemical properties of different tendons found variability within the tendon[37] as well as between tendons and ligaments.[1] Moreover, these results suggest that the patellar tendon may be a compositionally and/or functionally different tendon than the supraspinatus and Achilles tendons, which leads to an altered response to diabetes.

While this study provides insight into structural and mechanical alterations in a mouse model of type II diabetes, this study is not without limitations. While there have been some studies showing that diabetes is associated with altered fibril morphology and excess non-enzymatic collagen crosslinking, this study did not directly measure these structural parameters. Due to the role that collagen fibrils and GAGs are thought to play in tendon mechanical function, the study prioritized the determination of changes in those molecules. However, measurements of collagen cross-links or collagen fibril morphology in this model would also be very valuable. Since there are multiple hierarchical levels at which these morphological changes may affect tendon mechanics, these changes could be investigated in this particular mouse model in the future.

In addition, this study did not measure any morphometric parameters for the groups investigated. Animals in this study were fed the same diet and housed in the same conditions, and while no differences in activity were noted, activity levels were not explicitly monitored. Although not measured in this study, the db/db diabetic mice typically have a higher

body weight than the heterozygous mouse, which could lead to a more sedentary lifestyle for the diabetic mice.[4] Given that activity levels could be related to joint loading, we recognize this as a potential limitation of the study. However, we expect that the changes due to decreased activity would have a smaller effect on tendon mechanical function than the changes due to diabetes in this model.

Finally, this study only investigated one dynamic response of tendon to load, collagen fiber re-alignment. In order to provide a full description of tendon's response to load, it would be necessary to investigate other dynamic responses, such as uncrimping, fibril deformation, and fibril sliding, especially if the mechanical changes are expected to arise from changes in fibril structure or cross-linking. Despite these limitations, this study provides important data on the diabetic tendon response to load for three different tendons (Fig. 7). This study confirms that several measures of mechanical function are diminished in a mouse model of type II diabetes and adds evidence that collagen fiber re-alignment is also altered in this disease.

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